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Hydrocarbon-degrading filamentous fungi isolated from flare pit soils of northern and western Canada

by

Trevor Marc April

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

This study set out to isolate and identify filamentous fungi from flare pits across western and northern Canada and determine whether they possess the potential to degrade hydrocarbons while using this substrate as the sole carbon source. Sixty-four species of filamentous fungi were isolated, and tested using gas chromatography for their ability to degrade crude oil. Some of the isolates were further tested (using radiorespirometry) for their ability to mineralize aliphatic and aromatic hydrocarbons. Results indicate that the ability to degrade hydrocarbons is relatively widespread among species of filamentous fungi, being expressed in five families across four orders within the Ascomycota. A unique hydrocarbon-degrading ascomycete, *Pseudallescheria boydii*, exhibited considerable variability in morphology, colony appearance, colony diameter, and temperature tolerance among strains. Conspecificity among strains was supported by RFLP analysis of the ITS region of rDNA. The study indicates that many of the species isolated from flare pits may be important agents for *in situ* bioremediation of aliphatics in oil-contaminated sites.

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LIST OF ABBREVIATIONS AND SYMBOLS

Analytical Terms

GC - Gas chromatography

GC-MS - Gas chromatography - mass spectrometry

^{1}H NMR - Proton magnetic resonance spectroscopy

HPLC - High performance liquid chromatography

SEM - Scanning electron microscope

TLC - Thin-layer chromatography

Chemical Terms

ACS fluor - Aqueous counting scintillant fluor

B - Benomyl

C - Carbon

^{14}C - Radioisotope carbon

$CaCl_2 \bullet 2H_2O$ - Calcium chloride dihydrate

Carbo-Sorb - CO_2 trapping agent

CH_2Cl_2 - Dichloromethane

CO_2 - Carbon dioxide

$FeSO_4 \bullet 7H_2O$ - Ferrous sulfate heptahydrate

HCO_3^- - Bicarbonate

H_2SO_4 - Sulfuric acid

K_2HPO_4 - Di-potassium hydrogen orthophosphate

KH_2PO_4 - Potassium di-hydrogen orthophosphate

KNO_3 - Potassium nitrate

$MgSO_4 \bullet H_2O$ - Magnesium sulfate monohydrate

MOG - Mineral oil and grease

N_2 - Nitrogen gas

$NaHCO_3$ - Sodium bicarbonate

NaOH - Sodium hydroxide

n-C_n - Normal alkane

NH_4Cl - Ammonium chloride

NWC oil - Norman Wells crude oil

PAH - Polycyclic aromatic hydrocarbon

T - Oxytetracycline dihydrate

TEH - Total extractable hydrocarbons

µm - Micrometer

Culture Collections

ATCC - American Type Culture Collection

CCFC - Canadian Collection of Fungus Cultures

DAOM - Department of Agriculture, Ottawa

UAMH - University of Alberta Microfungus Collection and Herbarium

Measurement Terms

°C - Degrees Celsius

cm - Centimeter

dpm - Disintegrations per minute

g - Gram

hrs - Hours

L - Litre

M - Molar

mg - Milligram

min - Minute

mL - Millilitre

N - Normal

% - Percent

rpm - Revolutions per minute

w/v - Weight per volume

Media

CER - Cereal agar

CMA - Corn meal agar

CzA - Czapek's solution agar

MEA - Malt extract agar

MYB - Mycobiotic agar

MYPD - Malt extract, yeast extract, peptone and dextrose medium

OAS - Oil agar slurry

OAT - Oatmeal agar

PCA - Plate count agar

PDA - Potato dextrose agar

Molecular Terms

ITS region - Internally transcribed spacer region

PCR - Polymerase chain reaction

rDNA - Ribosomal DNA

RFLP - Restriction fragment length polymorphism

Study Sites

BL - Boundary Lake, British Columbia

DV - Drayton Valley, Alberta

KS - Kindersley, Saskatchewan

NW - Norman Wells, Northwest Territories

WG - Willesden Green, Alberta

Statistical Terms

$\leq$ - Less than or equal to

n - Sample size

SD - Standard deviation

Miscellaneous

cf. - Confer or compare

EPA - Environmental Protection Agency

N/A - Not available

SM - Standard methods

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The soil fungal community is a diverse assemblage of species that carries out a wide range of transformations of materials, from simple sugars, polysaccharides and polypeptides to complex polymers such as lignins and keratins. Understanding the full range of enzymatic activities of soil fungi is in part a function of knowing what species are present, because degradative capabilities are specific characteristics of species or even strains within a species. Selecting species with specific abilities to degrade pollutants is an important pursuit within the larger field of bioremediation and could lead to the discovery of taxa or assemblages of taxa with the ability to mineralize pollutants such as hydrocarbons.

The implementation of strict governmental regulations on oil-well site decommissioning over the last ten years has motivated gas and oil companies to consider a number of options when remediating and reclaiming sites. Concentrations of xenobiotic (man-made or unnatural chemical) contamination must be within certain residual levels established by the government prior to approval of site decommissioning. Among these sites are areas referred to as flare pits where petroleum wastes from gas and oil facilities are directed for disposal by pyrolization. Soils in these areas often become impregnated with complex petroleum compounds and derivatives, such as crude oil which is composed of several hundred constituents (Atlas 1981). These areas are particularly problematic for bioremediation because of the irregular consistency of soil

(variability of soil aggregates and porosity), uneven distribution and heterogeneity of contaminants and compound recalcitrance and toxicity (Skladany and Metting 1993).

Microbial degradation of petroleum pollutants has been the subject of many studies (Leahy and Colwell 1990) and could be a viable alternative to conventional techniques of remediation. However, the ways in which microorganisms, contaminants and the environment interact are not well understood. Further research is necessary to determine the role that microorganisms perform in bioremediation and the fate of contaminants in the environment (Atlas 1981). Substantial information has been collected about physical and chemical properties of contaminants found in the environment and of the environment itself, but enzymatic capabilities of indigenous microorganisms with respect to a number of contaminants, and toxicity of contaminants towards microorganisms, still require investigation. To stimulate effective biodegradation (particularly *in situ*), it is important to know which microorganisms are present and to understand their ability to degrade or utilize the contaminants.

Early studies on hydrocarbon-degrading fungi focused on filamentous soil fungi (Hyphomycetes; Davies et al. 1973; Davies and Westlake 1979; Flippen et al. 1964; Hoffman and Rhem 1976; Llanos and Kjoller 1976; Lowery et al. 1968; Markovetz et al. 1968; Nyns et al. 1968; Perry and Cerniglia 1973; and Zajic et al. 1969). In 1985, Bumpus et al. reported the oxidation of polycyclic aromatic hydrocarbons (PAHs) by the wood-decay basidiomycete *Phanaerochaete chrysosporium*, an observation that influenced the re-direction of research from hyphomycetes to basidiomycetes (white rot fungi). *P. chrysosporium* and other white rot fungi such as *Bjerkandera adjusta, Pleurotus ostreatus* and *Trametes versicolor* were shown to mineralize a range of PAHs

(Bezalel et al. 1996; Bumpus 1989; Bumpus and Aust 1987; de Jong et al. 1992; Field et al. 1992; George and Neufeld 1989; Hammel et al. 1986; Kotterman et al. 1994; Sutherland et al. 1993; Vazquez-Duhalt et al. 1994). The results from the research indicated that these fungi have potential as bioremediators of PAH-contaminated soils, thus solidifying the change of direction in research.

Several petroleum hydrocarbon degradation pathways have been well established. However, compounds in petroleum are not all utilized equally, an important consideration in bioremediation. The components of petroleum or crude oil can be divided into five fractions by liquid column chromatography: aliphatics, aromatics, polars, resins and asphaltenes. Simple linear aliphatics such as *n*-alkanes are most readily degraded while low-molecular-weight aromatics and branched and cyclic alkanes are more recalcitrant. High-molecular-weight aromatics, resins and asphaltenes are even more recalcitrant to microbial attack (Atlas 1981; Leahy and Colwell 1990). Metabolism of aliphatics and light aromatics (di- and tricyclic aromatics) by hyphomycete and zygomycete soil fungi is well known (reviewed by Cerniglia et al. 1985; Launen et al. 1995; Leahy and Colwell 1990; Oudot et al. 1993). In general, the greater the number of aromatic rings, the slower the rate of degradation (Kiehlmann et al. 1996). Gauger et al. (1989) observed that effective degradation of PAHs by fungi occurred when compounds had three or fewer rings, compared to PAHs containing four, five or six rings. However, some reports demonstrate that recalcitrant materials such as resins and asphaltenes can be co-oxidized if more utilizable hydrocarbons are available to support growth (Oudot et al. 1987; Perry 1979). Snellman et al. (1988) observed that many fungal isolates are able to degrade one type of crude oil, but were less efficient with other types where the constituents differed,

indicating substrate specificity. Davies and Westlake (1979) noted that the ability of certain fungi to grow on pure *n*-alkanes did not predict their ability to grow on crude oils. They also hypothesized that suppressed growth on crude oil may be more a function of the absence of utilizable compounds rather than a function of toxicity as was previously thought.

The term "degradation" or more specifically, "biodegradation," unfortunately has been used interchangeably with the terms "mineralization", "utilization" and "transformation." Biodegradation refers to the transformation of an organic compound to another form, without regard to the extent of the process (Sklandany and Metting 1993). Mineralization involves the complete degradation of an organic compound to carbon dioxide or inorganic molecules for the acquisition of energy. Mineralization may also infer utilization or assimilation where the compound is taken into the cells, undergoes enzymatic oxidation and is transformed into biomass, carbon dioxide, water and other inorganic molecules (Sklandany and Metting 1993). Partial degradation or transformation describes the fate of a compound that is modified, but not converted into inorganic compounds. Modifications range from simple oxidations or reductions or the loss of functional groups to more complex modifications where the compound is altered through a number of metabolic steps (Sklandany and Metting 1993). However, biodegradation of a compound is often used in the context of an organism's ability to grow on the compound, thus utilizing it in the process.

A number of filamentous fungi have been noted as efficient biodegraders of hydrocarbons (Kobayashi and Rittmann 1982). The ability to degrade hydrocarbons is widespread among the common genera of soil fungi with species of *Penicillium* and

Aspergillus being the most consistently reported (Davies and Westlake 1979; Kobayashi and Rittmann 1982; Launen et al 1995; Oudot et al. 1987, 1993; Nyns et al. 1968; Snellman et al. 1988; Wunder et al. 1994). A review by Leahy and Colwell (1990) reports that petroleum-degrading species of *Trichoderma* and *Mortierella* are among the most common soil isolates, with species of *Aspergillus* and *Penicillium* also being frequent. Chaineau et al. (1995), Lowery et al. (1968) and Davies and Westlake (1979) also described growth of *Trichoderma* isolates on hydrocarbons. Isolates of species classified in the Hyphomycetes as a "*Graphium*" sp. also have been noted as effective hydrocarbon-degraders (Hardison et al. 1997; Llanos and Kjoller 1976; Oudot et al. 1993; Zajic et al. 1969). Cerniglia et al. (1978) and Cerniglia and Gibson (1978, 1979, 1980) demonstrated the ability of the zygomycete *Cunninghamella elegans* to biodegrade both alkanes and aromatic compounds. Despite this ability, no studies have demonstrated that *C. elegans* is common in petroleum-contaminated soil. Table 1.1 summarizes species that have been reported in literature to degrade or grow on hydrocarbons.

Various methods have been used to determine whether filamentous fungi have the ability to degrade hydrocarbons. To isolate fungi with the ability to utilize gaseous hydrocarbons, Zajic et al. (1969) streaked raw sewage effluent on mineral salts medium flushed with a gaseous mixture composed of 40% natural gas. An unidentified species labelled "*Graphium*" sp. was isolated. Davies et al. (1973) conducted a similar study and observed that isolates of *Acremonium* spp., *Graphium* sp. and *Phialophora jeanselmei* could grow on gaseous *n*-alkanes. Flippen et al. (1964) isolated *Fusarium moniliforme* from diesel fuel by directly applying a small volume of contaminated fuel on mineral salts agar. *F. moniliforme* was further tested for its ability to utilize individual even-chain *n*-

alkanes (n-C_6 - n-C_{12}) and n-C_{13}. Growth of the fungus in this case was supported only by n-decane and n-dodecane.

A study by Markovetz et al. (1968) looked at the ability of fungi to assimilate alkanes and alkenes (C_{10} - C_{18}). They selected a group of fungi based on studies by Foster (1962), Krynitsky and McClaren (1962), Markovetz and Kallio (1964), Krause and Lange (1965) and Rynearson and Peterson (1965) that previously had demonstrated ability to grow on hydrocarbons. They assayed cultures in a basal salts medium to which one millilitre of a selected hydrocarbon had been added. The ability to utilize hydrocarbons was determined by observing mycelial growth on the various compounds tested. Growth response on each hydrocarbon was visually estimated and assigned to one of five categories ranging from "barely discernible" to "heavy". Strains of *Cunninghamella* (*C. blakesleeana* and an unidentified species) showed the best growth, followed by unidentified species of *Acremonium* and *Alternaria.* Strains of *Aspergillus fumigatus*, *A. niger*, and *A. terreus* and *Penicillium chrysogenum*, *P. notatum* and an unidentified *Penicillium* sp. varied in their ability to grow on hydrocarbons. Markovetz et al. (1968) also extracted metabolites with diethyl ether and assayed classes of compounds using thin-layer chromatography. The presence of a monocarboxylic acid, a primary alcohol and a secondary alcohol from a *C. blakesleeana* extract was detected.

Nyns et al. (1968) tried to assign a taxonomic value to the ability to assimilate hydrocarbons in sorting among taxa of filamentous fungi. Strains of unspecified origin were inoculated on slants of Yeast Nitrogen Base agar containing 2% sucrose. Spores or mycelium were then suspended in 5 ml sterile distilled water, collected and spread on the surface of slants of a similar medium containing 0.01% glucose. Fuel oil was added until

half the slant was submerged and mycelial growth below and above the level of the hydrocarbon was noted. These strains were screened for their ability to utilize fuel oil by doing a visual assessment of mycelial growth. A number of hyphomycetes and species from the Mucorales showed strong growth on the fuel oil. Species from the genera *Aspergillus*, *Fusarium* and *Penicillium* showed the most growth, however this ability was not consistent among all strains of any given species. Nyns et al. (1968) further tested these strains on individual hydrocarbons using the same method. Very few strains were capable of growth on *n*-alkanes of less than ten carbon atoms, whereas *n*-paraffins of 12 to 16 carbon atoms supported the best growth. They concluded that hydrocarbon assimilation is a strain-specific property and this ability can not necessarily be reported as a property of any given species or genus. This is an important point to consider when looking at bioremediation as a method to remediate soils: not all strains of a species reported to utilize hydrocarbons possess this ability. Therefore, although a species may be present in a sample, it does not indicate that it is utilizing hydrocarbons. The ability to assimilate or degrade hydrocarbons may depend on the substrate from which the strains are isolated. However, the source of the fungi tested by Nyns et al. (1968) was not documented.

Studies prior to the 1970s had focused primarily on the ability of known fungi to grow on hydrocarbons and little research was conducted on the isolation of hydrocarbon-degrading fungi naturally found in contaminated soils Furthermore, few studies had monitored the extent of alteration of the hydrocarbons after incubation with fungi. Early studies relied almost exclusively on visual assessments of mycelial growth of the strains they tested. Although growth can be a reliable indicator of hydrocarbon utilization, it is

difficult to determine which species are most efficient degraders. For example, when two species of *Penicillium* are grown on a specific hydrocarbon, one may normally develop floccose mycelium while the other produces lannose (low growing) mycelium. The species with the lannose growth habit may be a better degrader of that hydrocarbon, but based on growth response, the species with the floccose growth habit would appear to be a more efficient degrader. It is also difficult to use qualitative assessments of mycelial abundance to determine how many compounds an isolate is capable of utilizing when incubated on a complex hydrocarbon source such as crude oil. Nevertheless, these qualitative assessments can still be an effective way of screening for hydrocarbon degraders.

Studies in the 1970s continued with filamentous fungi, with more focus on species present in hydrocarbon-contaminated soils. Llanos and Kjoller (1976) conducted an experiment that involved applying oil to soil and comparing the composition of the fungal community to a control soil lacking hydrocarbons. Using dilution plating, they observed that a *Graphium* sp. and *Paecilomyces lilacinus* dominated the community in the oil-soaked soil during the first half of the one-year study. Neither was isolated from the control soil. Other species, in the genera *Acremonium*, *Mortierella*, *Gliocladium* and *Trichoderma*, dominated during the second half of the study period. This indicated that there was a succession of fungi in hydrocarbon-contaminated soils, presumably resulting from the sequential exhaustion of simple hydrocarbons. *Fusarium oxysporum* was observed throughout the duration of study as one of the dominant species, along with some *Penicillium* species. Llanos and Kjoller (1976) also measured growth response of the isolated fungi grown on individual *n*-alkanes (C_8 -C_{16}) and kerosene. Two

unidentified species of *Graphium*, *F. oxysporum*, *Penicillium nigricans*, *P. lilacinus* and *Acremonium sclerotigenum* all showed abundant growth. They concluded from this study that hydrocarbon-contaminated soils favor the growth of certain species and that the taxa frequently isolated in this environment are capable of utilizing crude oil and pure hydrocarbons.

Hoffmann and Rehm (1976) studied species from the Mucorales capable of degrading long chain *n*-alkanes. They looked at 21 species belonging to 11 genera which included strains isolated from hydrocarbon-contaminated soil and from culture collections. Assessments of growth were made on liquid basal salts medium with 0.01% yeast extract and on an agar (1.2%) medium, both amended with pure *n*-alkanes and with mixed *n*-alkanes. Hydrocarbons were mixed into the agar medium prior to solidification. They found that many species from the Mucorales grew well on solid media mixed with alkanes; however only *Absidia glauca*, *A. spinosa* and *Cunninghamella echinulata* grew well when inoculated into liquid media containing hydrocarbons. Species from the genera *Mortierella*, *Mucor*, *Rhizopus* and *Phycomyces* did not grow at all or showed limited growth on the side of the culture flask.

Davies and Westlake (1979) examined filamentous fungi they isolated from northern Canadian soils or obtained from culture collections. One gram of contaminated soil was added to a liquid mineral salts medium (pH adjusted to 4.6) with 0.2 mL crude oil, and incubated for up to two months without disturbance. Colonies were transferred to an aureomycin - rose bengal medium and eventually pure colonies were placed on malt agar until isolates were screened for their ability to utilize hydrocarbons using methods similar to Nyns et al. (1968; crude oil or *n*-tetradecane was used instead of fuel oil).

Growth on hydrocarbons was compared to oil-free inoculated controls. Spore suspensions from fungi that had grown on hydrocarbons were inoculated into a liquid mineral salts medium and incubated with 0.2 mL crude oil under static growth conditions. Hydrocarbon degradation was confirmed by gas chromatographic (GC) analysis of residual oil. Unidentified *Penicillium* spp. and *Verticillium* spp. were the most frequently isolated species and most showed the ability to degrade hydrocarbons. *Beauveria bassiana*, *Mortierella* sp., *Phoma* sp., *Scolecobasidium obovatum* and *Tolypocladium inflatum* were also observed to degrade hydrocarbons. *Verticillium* species were among the most efficient degraders, utilizing all the *n*-alkanes and almost completely degrading the isoprenoids pristane and phytane. *B. bassiana* and *S. obovatum* showed similar results, with *Penicillium* spp. and *T. inflatum* incompletely degrading most of the *n*-alkanes. Of the fungi received from culture collections, *Penicillium javanicum*, three unidentified *Penicillium* species, and an unidentified species each of *Phoma* and *Verticillium* showed the best growth on the various hydrocarbons tested. This study was one of the first to confirm degradation of hydrocarbons using an analytical method such as gas chromatography.

Hemida et al. (1993) mixed previously uncontaminated soil with benzene, kerosene or solar* at doses of 5% or 40% v/w and allowed samples to incubate for 60 days at 28°C. Fungi were then isolated by dilution plating on a glucose-Czapek's agar with rose bengal as a bacteriostatic agent and with or with out the addition of 1% hydrocarbon. They identified *Aspergillus* spp., *Cladosporium resinae*, *Emericella nidulans*, *Fusarium solani*, *Penicillium funiculosum*, *Rhizopus stolonifer* and *Trichoderma harzianum* as the most frequently isolated species. They equated the ability

to utilize hydrocarbons with frequency of isolation of species from hydrocarbon-contaminated soils; however no substrate analyses were performed. They concluded that the aforementioned species were all capable of utilizing benzene, kerosene or solar based on their isolation from soils contaminated with those hydrocarbons. However, these species are typically heavy sporulators and dormant spores in the contaminated soils may easily germinate under more suitable conditions such as on agar media. Most of the species isolated were more prevalent in the control soil than in the hydrocarbon-contaminated soil. Although these isolates may be capable of growth on hydrocarbons, no tests were performed to confirm this.

Oudot et al. (1987) isolated fungi from previously contaminated soils in France using a medium containing salts, chloramphenicol and crude oil for the specific isolation of

* The nature of this compound was not clarified by Hemida et al. (1996).

hydrocarbon-utilizing fungi. A malt agar medium was also used. Following the isolation of species, predominantly from the genera *Acremonium*, *Aspergillus*, *Fusarium*, *Gliocladium*, *Paecilomyces* and *Penicillium*, Oudot et al. (1987) analyzed their ability to attack various class fractions and individual compounds in crude oil using GC analysis and mass loss for each fraction. *Aspergillus flavus*, *A. fumigatus*, *Fusarium oxysporum*, *Penicillium janthinellum* and *P. thomii* were the most active species, preferentially degrading the aliphatic fraction; the aromatic fraction was the next most degraded. Asphaltene degradation by *F. oxysporum* was observed and *Aspergillus niger*, *A. flavus* and *F. oxysporum* also partially degraded the resins. Oudot et al. (1993) conducted a parallel study in Indonesia using similar methods. Comparable results were obtained,

with species of *Acremonium*, *Aspergillus*, *Emericella*, *Eupenicillium*, *Gliocladium*, *Graphium*, *Penicillium* and *Talaromyces* being the most common isolates. The most active species degrading the aromatic and aliphatic fractions were *A. fumigatus*, *Emericella nidulans*, *Eupenicillium javanicum* and *Graphium putredinis*. The resins were partially degraded by *Aspergillus flavipes*, *E. nidulans*, *E. javanicum* and *Gliocladium virens*. An unidentified *Acremonium* sp., as well as *E. nidulans*, *E. javanicum* and *G. putredinis* all partially degraded the asphaltenes. It is noteworthy that observed degradative capacities were not consistent within a species nor taxon-specific, a conclusion similar to that of Nyns et al. (1968).

Oudot et al. (1993) also compared degradation of petroleum between an individual species and combinations of active hydrocarbon-degrading strains and found them to be equally as efficient. This is an interesting observation because one would expect the mixed cultures to be more effective at degradation, degrading each class fraction of the oil to a greater extent. However, some of these fungi may exhibit antagonistic behaviour towards each other, thus reducing the overall level of degradation that would be expected.

A strain of *A. niger* was investigated for its ability to degrade pyrene (Wunder et al. 1994). Metabolites were isolated that included quinones, sulfate conjugates and phenols. The strain was isolated from PAH-contaminated soils and incubated in a mineral salts liquid medium with glucose and pyrene for 200 hrs. Radiorespirometry was employed to monitor the evolution of $^{14}CO_2$ and volatile metabolites. ^{14}C volatiles were trapped using 4 M KOH in one scintillation vial and benzethonium hydroxide in another, both placed in series, and headspace was flushed periodically with air. Metabolites were

analyzed and identified using reverse-phase high-performance liquid chromatography (HPLC), electron-impact mass spectrometry and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR).

Launen et al. (1995) completed a study that specifically examined the ability of nonbasidiomycetes to oxidize the large PAHs pyrene and benzo[a]pyrene. Fungi were isolated from soils contaminated with PAH levels ranging from 0.8 to 80 mg/g dry soil by sprinkling 0.1 g soil on MYPD medium (containing malt extract, yeast extract, peptone and dextrose) with the antibiotics penicillin and streptomycin sulfate. Fungi were screened for their ability to oxidize PAHs by adding a mycelial plug to MYPD liquid medium containing pyrene or benzo[a]pyrene. An uninoculated control flask was included. Thin-layer chromatography was used to measure levels of residual PAHs and to detect the major metabolites. Metabolites were identified by ^{1}H NMR and mass spectrometry. HPLC was also performed with cultures of *Penicillium janthinellum* using [^{14}C]-pyrene. They observed that over 50% of the isolates oxidized pyrene and benzo[a]pyrene. Of these, *Penicillium* species were the most common, and *P. janthinellum* and *Syncephalastrum racemosum* (Mucorales) showed the highest oxidation rates. *Fusarium* and *Trichoderma* species were also able to oxidize the PAHs. Launen et al. (1995) also reported that zygomycetes were isolated from areas with PAH levels less than 3 µg/g soil, suggesting that zygomycetes are less tolerant of oily wastes in high concentrations. This may explain why *Cunninghamella elegans* is not frequently isolated from hydrocarbon-contaminated soils.

Kiehlmann et al. (1996) continued along the same lines as Launen et al. (1995) examining polar metabolites from the biotransformation of chrysene by *Penicillium*

janthinellum, two unidentified *Penicillium* spp. and *Syncephalastrum racemosum*. Both studies used [^{14}C]-labeled compounds, a practice commonly used in studies of the hydrocarbon-degradative capacities of white rot fungi. In addition, they used proton magnetic resonance spectroscopy (^{1}H NMR), mass spectrometry, thin layer chromatography and HPLC to identify metabolites. Cerniglia (1982), Cerniglia et al. (1978, 1980, 1989), Cerniglia and Gibson (1977, 1979), and Cerniglia and Yang (1984) specifically studied *Cunninghamella elegans* and reported its ability to degrade a broad range of individual PAHs using methods similar to those of Kiehlmann et al. (1996) and Launen et al. (1995).

Studies of filamentous fungi and their abilities to degrade hydrocarbons have used qualitative assays of relative mycelial growth, measurement of residual hydrocarbons, and recently more analytical methods employing GC, mass spectrometry, HPLC and tracking radiolabeled substrates to determine the precise fate of substrate molecules.

Radiorespirometry is a method used to determine if a substrate is mineralized. Microorganisms are inoculated on liquid media and incubated with a [^{14}C]-labeled substrate in a sealed container. Following the incubation period, the medium is acidified (to shift the equilibrium from HCO_3^- to CO_2) and the gas headspace is flushed out with N_2 so that the $^{14}CO_2$ can be recovered with a CO_2-trapping agent. The presence of $^{14}CO_2$ infers that the [^{14}C]-labeled substrate is being utilized as a carbon and energy source and not transformed into other forms, as would occur if the substrate was merely being modified. To date, this technique has not been used extensively in studies examining the biodegradation of hydrocarbons by fungi, with Wunder et al. (1994) as one example. In general, fungi do not have the ability to oxidize PAHs to CO_2 when they are used as the

sole carbon source. The focus on PAH degradation has been whether PAHs can be transformed into less recalcitrant compounds, which may be more water-soluble and readily available for further metabolism.

Although the ability of filamentous fungi to degrade hydrocarbons appears to be widespread, few studies have dealt with the isolation of species from previously petroleum-contaminated soils. None has been conducted in the region studied in this thesis, with the exception of the work done by Davies and Westlake (1979) in the analysis of soils from Norman Wells, Northwest Territories. Also, the studies that did isolate fungi from contaminated soils identified only a few to the species level.

The primary hypothesis of this thesis is that there are naturally occurring fungal taxa in hydrocarbon-contaminated soils that have the ability to degrade hydrocarbons. The objectives set forth to test the hypotheses were: 1) to isolate and identify the mycota of hydrocarbon-contaminated soils; and 2) to test isolates for hydrocarbon-degrading ability using precise analytical techniques including GC and radiorespirometry.

Chapter 2 focuses on the fungal taxa isolated from hydrocarbon-contaminated soils and their ability to degrade hydrocarbons (crude oil). Taxa were isolated from five sites in a north to south transect from Norman Wells, Northwest Territories to Kindersley, Saskatchewan. All isolated fungi were tested using GC analysis for their ability to degrade a reference crude oil. A subgroup was chosen for in-depth study using radiorespirometry to determine if they mineralized crude oil.

The frequency of *Graphium* species appearance in literature and the presence of this hyphomycete in samples analyzed (Chapter 2) provided the incentive for a more in-depth study on this taxon in Chapter 3. This fungus was characterized in detail by

describing all stages of the life cycle including the sexual state, and by using restriction fragment length polymorphism (RFLP) analyses of the internally transcribed spacer (ITS) region or rDNA. A link to the Microascaceae was established when the teleomorph was identified as *Pseudallescheria boydii*. Its role as a potential agent for bioremediation of hydrocarbon-contaminated soils is discussed.

Chapter 4 is a synopsis of the potential of fungi as agents of bioremediation.

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Table 1.1. Reports in the literature of genera and species (Hyphomycetes, Ascomycetes and Zygomycetes) shown to utilize or degrade hydrocarbons.

Fungus	Reference
Hyphomycetes	
Acremonium sp.	Snellman et al. (1988); Davies et al. (1973)
A. kiliense	Snellman et al. (1988)
A. strictum	Chaineau et al. (1995); Oudot et al. (1987); Snellman et al. (1988)
A. sclerotigenum	Llanos and Kjoller (1976)
Alternaria sp.	Snellman et al. (1988)
Aspergillus sp.	Kobayashi and Rittmann (1982); Snellman et al. (1988); Nyns et al. (1968)
A. alliaceous	Chaineau et al. (1995); Lowery et al. (1968)
A. candidus	Chaineau et al. (1995); Lowery et al. (1968)
A. chevalieri	Snellman et al. (1988)
A. exidicaulus	Chaineau et al. (1995)
A. ficuum	Snellman et al. (1988)
A. flavipes	Oudot et al. (1993)
A. flavus	Oudot et al. (1987, 1993); Lowery et al. (1968)
A. fumigatus	Chaineau et al. (1995); Oudot et al. (1993); Oudot et al. (1987); Snellman et al. (1988)
A. heteromorphus	Snellman et al. (1988)
A. japonicus	Sahasrabudhe et al. (1985)
A. niger	Cerniglia (1984); Chaineau et al. (1995); Oudot et al. (1987); Snellman et al. (1988); Cerniglia et al. (1978)
A. ochraceous	Cerniglia (1984)
A. repens	Snellman et al. (1988)
A. ruber	Snellman et al. (1988)
A. sydowii	Oudot et al. (1993); Snellman et al. (1988)
A. tamarii	Oudot et al. (1987)
A. terreus	Snellman et al. (1988)
A. ustus	Snellman et al. (1988)
A. versicolor	Snellman et al. (1988); Perry and Cerniglia (1973)
Beauveria bassiana	Davies and Westlake (1979)
Aureobasidium sp.	Davies and Westlake (1979)
Cephalosporium roseum	Lowery et al. (1968)
C. acremonium	Perry and Cerniglia (1973)
Chrysosporium sp.	Davies and Westlake (1979)
Cladosporium herbarum	Chaineau et al. (1995)
C. cladosporioides	Snellman et al. (1988)
Curvularia lunata	Cerniglia (1984)
Dematium gaugertii	Lowery et al. (1968)
Epicoccum nigrum	Cerniglia (1992)
Fusarium sp.	Lowery et al. (1968); Kobayashi and Rittmann (1982); Nyns et al. (1968)
F. moniliforme	Flippen et al. (1964)
F. oxysporum	Oudot et al. (1987); Llanos and Kjoller (1976)
F. solani	Chaineau et al. (1995)
Gliocladium sp.	Cerniglia (1984)
Geomyces pannorus	Cerniglia et al. (1992)
G. catenulatum	Lowery et al. (1968)
G. roseum	Oudot et al. (1987)
Gongronella butleri	Chaineau et al. (1995)
Graphium sp.	Llanos and Kjoller (1976); Snellman et al. (1988); Zajic et al. (1969); Davies et al. (1973)
G. fructicolum	Llanos and Kjoller (1976)
G. rubrum	Lowery et al. (1968)
G. putredinis	Oudot et al. (1993)
Humicola fuscoatra	Snellman et al. (1988)

Mortierella sp.	Davies and Westlake (1979)
Oidiodendron sp.	Nyns et al. (1968)
Paecilomyces sp.	Lowery et al. (1968)
P. lilacinus	Oudot et al. (1987); Llanos and Kjoller (1976)
P. variotii	Lowery et al. (1968)
Penicillium spp.	Lowery et al. (1968); Kobayashi and Rittmann (1982); Snellman et al. (1988); Davies and Westlake (1979); Nyns et al. (1968)
P. chrysogenum	Cerniglia (1984); Snellman et al. (1988); Cerniglia et al. (1978)
P. citrinum	Oudot et al. (1993); Snellman et al. (1988)
P. herquei	Snellman et al. (1988)
P. janthinellum	Kiehlmann et al. 1996; Launen et al. 1995; Oudot et al. (1987); Snellman et al. (1988)
P. nigricans	Llanos and Kjoller (1976)
P. notatum	Cerniglia (1984)
P. ochro-chloron	Cerniglia (1984); Perry and Cerniglia (1973)
P. oxalicum	Snellman et al. (1988)
P. pinophylum	Chaineau et al. (1995)
P. thomii	Oudot et al. (1987)
P. zonatum	Perry and Cerniglia (1973)
Phialophora jeanselmei	Davies et al. (1973)
Scolecobasidium obovatum	Davies and Westlake (1979)
Tolypocladium inflatum	Davies and Westlake (1979)
Trichoderma sp.	Lowery et al. (1968)
T. harzianum	Chaineau et al. (1995)
T. koningii	Chaineau et al. (1995)
T. pseudokoningii	Chaineau et al. (1995)
T. viride	Davies and Westlake (1979)
Verticillium sp.	Davies and Westlake (1979)
Ascomycetes	
Emericella nidulans	Hemida et al (1993); Oudot et al. (1993)
Eupenicillium javanicum	Oudot et al. (1993)
Neurospora crassa	Cerniglia (1992)
Sordaria funicola	Cerniglia (1992)
Talaromyces bacillisporus	Oudot et al. (1993)
Zygomycetes	
Absidia sp.	Cerniglia (1984); Cerniglia et al. (1978); Hoffmann and Rehm (1976)
A. glauca	Cerniglia (1984); Cerniglia et al. (1978)
A. pseudocylindrospora	Cerniglia (1984)
A. ramosa	Cerniglia (1984)
A. spinosa	Cerniglia (1984)
Basidiobolus ranarum	Cerniglia (1984)
Circinella sp.	Cerniglia et al. (1978)
Circinella umbellata	Hoffmann and Rehm (1976)
Choanephora campincta	Cerniglia et al. (1978)
Cokeromyces poitrassi	Cerniglia et al. (1978)
Conidiobolus sp.	Cerniglia et al. (1978)
Cunninghamella elegans	Cerniglia (1984); Kobayashi and Rittmann (1982); Snellman et al. (1988); Cerniglia et al. (1978); Perry and Cerniglia (1973)
C. bainieri	Cerniglia (1984)
C. blakesleeana	Markovetz et al. (1968); Cerniglia (1984)
C. echinulata	Cerniglia (1984); Cerniglia et al. (1978)
C. japonica	Cerniglia et al. (1978)
Gilbertella persicaria	Cerniglia (1984); Cerniglia et al. (1978)
Mortierella verrucosa	Cerniglia (1992)
Mucor sp.	Cerniglia (1984); Snellman et al. (1988); Cerniglia et al. (1978); Hoffmann and Rehm (1976)
M. abundans	Lowery et al. (1968);
M, genevensis	Lowery et al. (1968)
M. hiemalis	Cerniglia (1984); Cerniglia et al. (1978); Hoffmann and Rehm (1976)
Phycomyces blakesleeanus	Cerniglia (1984); Hoffmann and Rehm (1976)

Rhizopus arrhizus	Cerniglia (1984); Hoffmann and Rehm (1976)
R. stolonifer	Cerniglia (1984)
R. oryzae	Cerniglia (1992)
Smittium culisetae	Cerniglia (1984)
S. culicis	Cerniglia (1984)
S. simulii	Cerniglia (1984)
Syncephalastrum sp.	Cerniglia (1984); Cerniglia et al. (1978)
S. racemosum	Cerniglia (1984); Snellman et al. (1988); Cerniglia et al. (1978)
Thamnidium elegans	Cerniglia (1984); Hoffmann and Rehm (1976)
Zygorhynchus moelleri	Cerniglia (1984); Hoffmann and Rehm (1976)

CHAPTER 2

Filamentous fungi isolated from flare pits in northern and western Canada with the ability to degrade hydrocarbons

INTRODUCTION

The soil fungal community consists of a diverse group of saprotrophic species with various enzymatic capabilities, permitting them to obtain carbon and nitrogen from a variety of sources including simple C_5 and C_6 sugars, amino acids and complex polymers such as proteins, as well as cyclic compounds such as lignins and tannins (Dix and Webster 1995). Fungi are also capable of utilizing a variety of substrates present as contaminants of industrial origin which encompass a series of materials with naturally occurring analogues such as aliphatic compounds and aromatic aldehydes and acids (Dix and Webster 1995). To understand and ultimately deploy the enzymatic activies of the fungal soil community, it would be useful to know what species are present because degradation capabilites are specific characteristics of species or even strains within a species. Selecting species with specific abilities to degrade pollutants is an important pursuit within the larger field of bioremediation and could lead to the discovery of taxa or assemblages of taxa with the ability to mineralize pollutants such as hydrocarbons.

The extraction of natural gas and petroleum from many sites over the last half-century in western and northern Canada has resulted in petroleum-contaminated soils. Among these sites are areas referred to as "flare pits" where petroleum wastes from gas and oil facilities are dumped and burned. The soils in these pits become laden with

complex petroleum compounds and salts from brines used during the drilling process and are particularly problematic for bioremediation because of the diversity, persistence, and often toxic nature of the suite of compounds that accumulate here (Skladany and Metting 1993). In addition to having a wide range of enzymatic activities, filamentous fungi can serve as oligotrophs and grow in environments with very low concentrations of carbon and nitrogen, using their mycelial network to transport limiting nutrients from one area to another (Dix and Webster 1995). Some groups of fungi, such as the Eurotiales, are also osmo- and xerotolerant and are able to grow in soils with low water availability or high salt concentrations. For these reasons, fungi have considerable potential for bioremediating petroleum-contaminated soils.

Studies from the late 1960s and early 1970s demonstrated that a number of filamentous fungi have the ability to grow on hydrocarbons, with *Aspergillus* and *Penicillium* species being the most commonly isolated genera (Davies et al. 1973, Davies and Westlake 1979; Flippen et al. 1964; Hoffmann and Rhem 1976; Llanos and Kjoller 1976; Lowery et al. 1968; Markovetz et al. 1968; Nyns et al. 1968; Perry and Cerniglia 1973; Zajic et al. 1969). In spite of these observations and others of a similar nature (Cerniglia 1986, 1989, 1990; Hemida et al. 1993; Kiehlmann et al. 1996; Launen et al. 1995; and Oudot et al. 1987, 1993), a study (Bumpus et al. 1985) showing that white-rot basidiomycetes could oxidize PAHs caused a major shift in emphasis toward *Phanerochaete chrysosporium* and other wood-decay basidiomycetes (e.g., *Bjerkandera adjusta, Pleurotus ostreatus* and *Trametes versicolor*) (Bezalel et al. 1996; Bumpus 1989; Bumpus and Aust 1987; de Jong et al. 1992; Field et al. 1992; George and Neufeld 1989;

Hammel et al. 1986; Hammel et al. 1992; Sutherland et al. 1990; and Vazquez-Duhalt et al. 1994) for bioremediation of hydrocarbons.

In oil-producing areas in northern and western Canada, little is known about the indigenous fungi present in contaminated soils or their ability to utilize hydrocarbons. This study set out to select filamentous fungi from flare pits and to determine whether they possess the potential to degrade hydrocarbons while using them as the sole carbon source. Five flare pit sites were chosen, one from the Northwest Territories, three from central Alberta/ British Columbia, and one from southwestern Saskatchewan. Sixty-four species of filamentous fungi were isolated and identified and tested for their ability to grow on crude oil as a sole carbon source. Isolates that demonstrated growth on crude oil were tested for their ability to degrade hydrocarbons using gas chromatography. A select group of 23 isolates was chosen and tested (using radiorespirometry) for their ability to mineralize aliphatic and aromatic hydrocarbons.

MATERIALS AND METHODS

SITE DESCRIPTION

Hydrocarbon-contaminated soils were collected from five flare pits (Fig. 2.1). The most northern site, Norman Wells (NW), is located in the McKenzie River valley, in an area of muskeg and forest dominated by *Picea mariana*. The Drayton Valley (DV) site is situated in a forested region dominated by *Populus tremuloides*, *Pinus contorta* and *Picea glauca*. Boundary Lake (BL) and Willesden Green (WG) sites are surrounded by cropland with *Populus tremuloides* the most prevalent tree although stands of *Picea*

glauca are also in the vicinity of Willesden Green. The most southerly site is Kindersley (KS), near the western border of Saskatchewan. This site is situated amidst cropland in a prairie grassland region with stands of *Populus tremuloides* nearby.

Soil analyses

Soil analyses were performed by Phillip Analytical Services Corp., Burnaby, BC. Permission for use of soil data in this study was granted by Imperial Oil Resources Ltd. Mineral oil and grease (MOG) content was determined using Standard Methods (1989) protocol SM18 5520 C,D&F; Total oil and grease was determined using Environmental Protection Agency protocol EPA 9071A (Grav.) and SM18 5520 D (TR) protocol; Total polycyclic aromatic hydrocarbon (PAH) content was determined using EPA 3540B/8270 (Mod.) protocol; and Total extractable hydrocarbon (TEH) content was determined using EPA 8015 (Mod.) protocol.

Isolation and Culture Methods

Two samples of soil were collected from visibly petroleum-contaminated patches of soil at each flare pit. Soil samples within the flare pit were chosen. Samples were collected from the top 10 cm layer using alcohol-sterilized spoons and stored in two 500 mL sterile glass jars in the dark at 4 ℃ until plated.

Fungi were isolated by dilution plating and direct plating. In the dilution method, 20 g of soil was added to 180 mL of sterile twice-distilled water and homogenized in a sterile, metal canister for 30 seconds. Ten millilitres of the soil solution was transferred with a sterile pipette into a 200 mL Erlenmeyer flask containing 90 mL of sterile twice-

distilled water to yield a 100-fold dilution. Three-tenths of a millilitre of the soil solution was then pipetted onto two plates of each medium and spread using sterile bent glass rods. In the second plating method, approximately 5 g of soil was sprinkled onto two plates of each medium before the medium had completely solidified. Both dilution and sprinkle plates were incubated in the dark at room temperature (~22 °C) until fungal colonies developed. Pure colonies were established from the isolation plates by transferring conidia or mycelia to MEA+T (see below) using a single point inoculum.

Strains of fungi from DV, WG and BL were isolated on four culture: malt extract agar (MEA+T) - 15 g malt extract (Difco), 15 g Bacto-agar (Difco), and 0.1 g oxytetracycline dihydrate (T; Sigma); MEA+T+B, malt extract agar, oxytetracycline and 2 μg benomyl (B; Sigma); corn meal agar (CMA+T) - 17 g corn meal agar (Difco) and oxytetracycline; and mycobiotic agar (MYB) - 35.6 g mycobiotic agar (Difco). All media were prepared per Litre twice-distilled water. A 1% (w/v) stock solution of benomyl was prepared by adding 2 g benomyl to 200 mL acetone; 0.2 mL (= 2 μg) of stock was added per Litre medium. For soils from NW and KS, CMA+T was replaced with Oil Slurry agar (OSA) - 4 g Bacto-agar and 6% v/v Norman Wells Crude (NWC) oil. OSA was prepared by aliquoting 15 mL warm (~60^{0}C), sterilized agar into 25 mL sterile screwcap test tubes and adding 1 mL NWC oil. Tubes were shaken vigorously by hand and poured immediately into sterile Petri plates. Fifty microlitres of heat-sterilized nutrient solution containing 25.0 g K_2HPO_4, 50.0 g NH_4Cl, and 100.0 g KNO_3 (per Litre twice-distilled H_2O) was then spread on the surface of each plate containing solidified OAS. Oxytetracycline was not added to OAS because it was assumed that only a few bacterial

species would be able to grow directly on the oil and those would not interfere with growth of fungi or with attempts to isolate fungi.

Strain Identification and Phenotype Testing

Colony colour, morphology and growth rate on MEA were recorded. Growth rate and macroscopic features of *Aspergillus* and *Penicillium* isolates were also observed on a modified Czapek's solution agar [CzA; Pitt 1985; 35 g Czapek Dox broth (Fischer Scientific), 1 g K_2HPO_4, 0.01 g $ZnSO_4 \cdot 7H_2O$, 5 g yeast extract, and 15 g Bacto-agar]. *Fusarium* species were observed on Oatmeal Agar (OAT; Weitzman and Silva-Hutner 1967). Conidiogenesis was examined using slide culture preparations (Sigler 1992). To encourage sporulation, slide cultures were prepared with cereal agar (CER, Padhye 1973) and *Fusarium* spp. were incubated under black light (Nelson et al. 1983). Species identifications were based on morphological criteria only according Barron (1968), Carmichael et al. (1980), de Hoog (1977), Domsch et al. (1980), Ellis (1971, 1976), Klich et al. (1988), Nelson et al. (1983), Pitt (1985), Ramirez (1982) and Samson (1974). Species analyzed by radiorespirometry (below) are annotated and described in Appendix A. Because isolates of *Aspergillus* and *Penicillium* were difficult to identify to species, they are also described in Appendix A.

Fungal isolates were tested for their ability to utilize crude oil using growth response as an indicator. All isolates were inoculated onto 20 mL mineral salts agar (Chapter 3; Bacto agar was substituted for Noble agar). Isolates were inoculated in duplicate and incubated at room temperature (~22°C) for one week to establish mycelial growth, at which time 0.050 mL nutrient solution was added to both plates and allowed to

penetrate into the agar. Subsequently, 0.1 mL NWC oil was added to only one plate. Following a 14 day incubation period in the dark, growth was compared between the two plates. Cultures incubated with NWC oil showing significantly heavier conidiogenesis, greater colony diameter or more abundant mycelium than cultures without oil were further examined further for their ability to degrade hydrocarbons using the techniques described below.

Analytical Methods

Capillary Gas Chromatography

Isolates were inoculated onto mineral salts agar medium in 15 mm x 100 mm glass Petri plates and incubated with NWC oil and nutrient solution similar to that described above to screen for hydrocarbon-degrading ability. Sterile, uninoculated controls were included for comparison. Cultures were incubated for 14 days at room temperature in the dark. On Day 14, 0.025 mL stock surrogate standard solution (containing chrysene and squalane) was added, and residual oil was recovered in pentane (HPLC grade; Caledon Laboratories Ltd.) and prepared for gas chromatographic (GC) analysis using the methods outlined in Chapter 3, without liquid chromatographic fractionation. Samples were analyzed by GC using a Hewlett Packard Model 5890 chromatograph fitted with a DB-5 fused silica column (J + W Scientific), 25 m x 0.2 mm. The temperature program used by Foght et al. (1990) of 90°C for 2 min, increasing at 4°C/min to 250°C and held for 18 min, was followed. The degree of hydrocarbon degradation was scored as: "no degradation", "partial degradation" (slight degradation of *n*-alkanes; *n*-C_{12} - *n*-C_{26} range), "good degradation" (significant degradation of *n*-alkanes,

but not the isoprenoids or branched and cyclic alkanes) or "significant degradation" (significant degradation of all alkanes). The isoprenoids pristane and phytane (15 carbon-unit alkanes with four and five methyl substitutions, respectively) are relatively resistent to microbial degradation, and are often used as a measure of aliphatic degradation.

Following GC analysis, selected isolates were inoculated in triplicate onto mineral salts agar and incubated with NWC oil as the sole carbon source. After incubation and recovery of the residual oil, samples were fractionated (liquid column chromatography) essentially by the method of Fedorak and Westlake (1981; modified to exclude the activated granular copper layer) and each fraction was analyzed by GC.

Radiorespirometry

Sixteen species (23 isolates; Table 2.3) were chosen for radiorespirometric study based either on prevalence at each site or on GC profiles indicating significant degradation of the crude oil, with at least one randomly selected isolate used to represent each species. To determine whether the isolates were capable of mineralizing compounds in the saturate and aromatic fractions of crude oil, isolates were incubated with NWC spiked with *n*-[1-^{14}C]-hexadecane or [9-^{14}C]-phenanthrene (Amersham Corporation, Arlington Heights, IL). These compounds were chosen as representatives of the alkane and aromatic fractions, respectively. The radiolabeled compounds were added individually to NWC oil to provide 1 - 4 x 10^6 disintegrations per minute (dpm) per mL oil, accurately determined (Foght et al. 1990).

Originally, conidia or mycelia collected from cultures with an inoculation probe were used as inocula. However, inconsistent $^{14}CO_2$ production among triplicate samples

of some isolates lead to the development of a more standard inoculation procedure. MEA plugs (6 mm diameter) were taken from the perimeter of 7 day old colonies and placed into 158 mL serum bottles, in triplicate, containing 20 mL liquid mineral medium (i.e., mineral salts lacking agar). Isolates were incubated at room temperature (~22°C) on a rotary shaker (200 rpm) for one week to establish mycelial growth. At that time (Day 0), 0.050 mL sterile nutrient solution was added to all inoculated bottles and to three sterile control bottles. After 0.100 mL NWC oil containing [^{14}C]-hexadecane had been added to provide 2.34 x 10^5 dpm per bottle, serum bottles were sealed with rubber caps and incubated for 14 days on a rotary shaker (200 rpm) in the dark at room temperature.

On Day 14, 0.1 mL of culture medium was withdrawn aseptically from each bottle with a sterile syringe (22 gauge x 3.9 cm) and plated on plate count agar (PCA; Difco; 38.0 g/L) to check for purity (i.e., bacterial contamination). Each bottle was then acidified with 1 mL 4N H_2SO_4 (to pH $\leq$2) and $^{14}CO_2$ recovered using methods similar to Fedorak et al. (1982). Bottles were flushed by bubbling N_2 through the medium at 100 mL/min for 10 min using a 20 gauge x 15.2 cm needle. The gas flow carried the CO_2 through two scintillation vials in series, each containing 1 mL of CO_2 trapping agent (Carbo-Sorb; Packard Instrument Co., Downover Grove, IL) in 10 mL ACS fluor (Amersham Corp., Arlington Heights, IL). All samples were counted on a Beckman LS 3801 scintillation counter with quench correction. The percent ^{14}C as $^{14}CO_2$ was calculated based on the original amount of ^{14}C added as *n*-[1-^{14}C]-hexadecane.

The CO_2 trapping efficiency of the system was determined using a method adapted from Fedorak et al. (1982). To a 158 mL serum bottle containing 20 mL liquid mineral salts medium, 0.150 mL $NaH^{14}CO_3$ (2.05 x 10^5 dpm) in 0.025 M borax buffer (pH

8.6) was added. The bottle was stoppered and the solution acidified with 2 mL 4 N H_2SO_4 and flushed with N_2 as described above. This test was repeated four times, and the trapping efficiency was determined to be 101.5% ± 0.78%. A background vial, containing fluor and Carbo-Sorb, was counted each time and that value (typically 20±5 dpm) was subtracted from the value obtained for each test vial before calculating percent recovery.

Following flushing of CO_2, residual ^{14}C in two randomly selected serum bottles was recovered in three fractions: organic, aqueous, and hyphal biomass (Fig. 2.2). Two millilitres 2 M NaOH was added to neutralize each culture and hyphal biomass was recovered by filtering the culture medium through tightly packed glass wool in a funnel, and collecting the filtrate in a 50 mL separatory funnel. The organic fraction (residual oil, unaltered labeled compound and uncharged organic metabolites) was extracted from the aqueous fraction by adding ≤10 mL pentane to the 50 mL separatory funnel and gently swirling. The aqueous fraction was collected in a volumetric flask and the volume was adjusted to 25 mL with double-distilled water. The organic fraction was collected in a volumetric flask and the volume adjusted to 10 mL with pentane. One millilitre aliquots of each fraction were counted in 10 mL ACS fluor. The glass wool with entrained cell biomass was rinsed with 2 mL CH_2Cl_2 to remove ^{14}C-labeled oil or metabolites adhering to the mycelium. The CH_2Cl_2 rinse was placed in a scintillation vial containing 10 mL ACS fluor; similarly, the glass wool with entrained hyphae was placed into a scintillation vial. All samples were counted on a Beckman LS 3801 scintillation counter with quench correction.

Isolates were also incubated in liquid culture with unlabeled NWC oil to compare GC profiles of residual oil to profiles of residual oil recovered from cultures grown on solid medium. Cultures were acidified and residual oil was extracted by adding 10 mL CH_2Cl_2 to bottles, gently swirling, and filtering the contents through glass wool into a 50 mL separatory funnel. The organic fraction containing residual oil was separated and dried by filtering through anhydrous Na_2SO_4. CH_2Cl_2 was evaporated using a stream of N_2 and replaced with 5 mL pentane for GC analysis.

Isolates that demonstrated levels of mineralization greater than 5% in the *n*-[1-^{14}C]-hexadecane mineralization study were chosen for incubation with [9-^{14}C]-phenanthrene in NWC oil using the same method. One-tenth millilitre of NWC oil mixed with [9-^{14}C]-phenanthrene was added to each bottle to provide 1.56×10^5 dpm per bottle. CO_2 was recovered and filtrate was separated into different fractions to recover residual ^{14}C (Fig. 2.2).

RESULTS

SOIL ANALYSIS

Limited soil data were available for each site and none were available for soils from NW. Because soil analyses were not conducted at the same time, similar tests were not completed on all soils and it is difficult to compare data among the sites. MOG values (62 000 - 130 000 mg/kg soil) and pH (8.6) were reported only for KS and total PAH was determined only for WG (19.68 - 68.67 mg/kg soil). TEHs (C_{10} - C_{30}) were highest in

WG at 216 400 mg/kg soil; BL and DV had values of 84 000 and 48 000 - 78 000 mg/kg soil, respectively. The TEH content for KS was measured from C_{10} - C_{60} and determined to be 57 000 mg/kg soil. For TEH values C_{30} or higher, DV had the highest values ranging from 14 000 - 29 000 mg/kg soil; BL had a value of 16 000 mg/kg soil and had a PAH content ranging from 19.68 - 68.67 mg/ kg soil. BL and DV soils were composed of sandy clay and KS was composed of silty clay; soil lithology was not available for WG.

DISTRIBUTION OF FUNGAL ISOLATES

Over 400 fungal isolates representing at least 64 species were obtained from flare pit soils contaminated with hydrocarbons. Molds (Hyphomycetes) and ascomycetes were the most common. Very few zygomycete isolates (represented by one species, *Rhizopus stolonifer*) were observed (BL and KS). No basidiomycetes were identified. Two species of *Phoma* (Coelomycetes) were observed. Table 2.1 summarizes the species that were frequently isolated, the site(s) from which they were obtained, the method and media used to isolate them, and their ability to grow on hydrocarbons as the sole carbon source. Growth on mineral medium with crude oil was compared with growth on hydrocarbon-free mineral medium. GC results are summarized in Table 2.1 to indicate the ability of each species to degrade crude oil (see below). Sterile fungi, uncommon species or contaminated isolates are not included in the table. Species determinations for *Aspergillus* and *Penicillium* isolates were difficult because of subtle differences between species, and in instances where identifications were uncertain, the closest name was given to the species they resembled. These are designated by the abbreviation cf. (= confer or

compare). Species of *Aspergillus* and *Penicillium* and isolates analyzed for their ability to mineralize crude oil (Table 2.3) are annotated in Appendix A. Annotations include descriptions of colony colour and morphology, conidiophores and conidia. Ambiguous characters which complicated species identification are discussed.

Species common to all or most sites included *Aspergillus niger*, *Gliocladium* cf. *catenulatum*, *Penicillium* cf. *janthinellum* and *Pseudallescheria boydii*. Species of *Trichoderma* were also frequently isolated from all sites and were observed using both isolation methods and on all media. However, isolates of *Trichoderma* which produce abundant sticky masses of conidia were discarded because of their potential to contaminate other isolates.

Looking specifically at each site (Table 2.2), *Pseudallescheria boydii* was amongst the most common species in the NW flare pit along with *Aspergillus niger*, *Gliocladium* cf. *catenulatum*, *Neosartorya fischeri*, *Penicillium* cf. *glabrum*, *Penicillium* cf. *janthinellum*, *Penicillium* cf. *purpurogenum*, *Penicillium thomii* and two unidentified *Penicillium* spp. *Phialophora americana*, *Phialophora verrucosa* and *Acremonium* spp. were also abundant, but less common.

Among the isolates from the three central study sites (BL, DV and WG), the most common were species of *Penicillium*. BL isolates included *Penicillium* cf. *janthinellum*, *Penicillium* cf. *miczynaskii*, *Penicillium thomii*, and four unidentified species of *Penicillium*. *Fusarium subglutinans*, an unidentified *Fusarium* sp. and *Oidiodendron griseum* and were also relatively common. *O. griseum* was not observed at any other site. DV soils yielded the greatest richness of *Penicillium* species, represented by *Penicillium* cf. *decumbens*, *Penicillium* cf. *glabrum*, *Penicillium* cf. *isariiforme*, *Penicillium* cf.

janthinellum, *Penicillium* cf. *purpurogenum*, *Penicillium spinulosum*, *Penicillium thomii*, *Penicillium* cf. *waksmanii* and seven unidentified species of *Penicillium*. *Fusarium* species, such as *Fusarium avenaceum*, *Fusarium subglutinans* and two unidentified species were also common along with *Acremonium butyri*, *Acremonium kiliense*, *Acremonium strictum*, *Paecilomyces variotii* and *Pseudallescheria boydii*. *Beauveria bassiana*, *Exophiala jeanselmei*, *Paecilomyces marquandii* and *Sporothrix schenckii* were unique to this site. The most common isolates in the WG soil include *Aspergillus fumigatus*, *Fusarium avenaceum*, *Fusarium subglutinans*, *Penicillium* cf. *janthinellum*, *Penicillium* cf. *purpurogenum*, *Penicillium thomii*, four unidentified *Penicillium* spp., and *Pseudallescheria boydii*. *Aspergillus fumigatus*, *Penicillium* cf. *janthinellum* and *Penicillium thomii* were the most common. *Geomyces pannorus* and *Paecilomyces variotii* were also isolated, but were not frequently isolated. *Gliocladium* cf. *catenulatum* was a very common isolate from soils from all sites.

Aspergillus and *Penicillium* species were the most common among the isolates from the most southerly site (KS). Few other species were frequently isolated from this site. *Aspergillus* species included *Aspergillus* cf. *niveus*, *Aspergillus* cf. *tamarii* and two unidentified *Aspergillus* spp. *Penicillium* species included *Penicillium* cf. *aurantiogriseum*, *Penicillium minioluteum*, *Penicillium pinophilum*, *Penicillium.* cf. *variabile* and two unidentified *Penicillium* spp. *Penicillium* cf. *variabile* was the most common species at this site.

The recovery of species was best on MEA+T medium, with growth of almost all species observed on this isolation medium. MEA+T+B medium was more selective and species from the genera *Aspergillus*, *Beauveria*, *Phialophora* and *Rhinocladiella* were

more common on this medium than on others. The smallest number of species was isolated on CMA+T medium. In many instances, this medium would become overgrown with species of *Gliocladium*, *Penicillium* and *Trichoderma*, thus limiting successful isolation of slower growing species. *Neosartorya fischeri*, *Oidiodendron griseum* and *Paecilomyces variotii* were some of the species readily isolated on this medium. MYB containing cycloheximide reduced the overall number of *Penicillium* isolates. *Pseudallescheria boydii* was one of the more common species on this medium.

A variety of species were isolated on OAS medium, though the most frequently isolated species belonged to the genus *Penicillium*. One difficulty with this medium is that many of the isolates obtained produced only vegetative mycelium with very few developing conidiophores; this was most noticeable on dilution plates. Transfer of vegetative hyphae from OAS to MEA often resulted in appearance of several different species that required further purification.

More species were isolated using the direct plating method than dilution plating (Table 2.1). Many species that readily produce small conidia were prevalent on dilution plates and were also present on direct (sprinkle) soil plates, with additional species being present.

GROWTH ON HYDROCARBONS

Fungal isolates were screened qualitatively for their ability to grow on crude oil as the sole carbon source. The criterion used was the production of visibly more mycelial mass or conidiogenesis on mineral medium with crude oil compared to growth on mineral medium without oil. Table 2.1 summarizes the ability of isolates to grow on NWC oil.

Some species of *Penicillium* and *Aspergillus* sporulated only slightly better on mineral medium with crude oil. Mycelium grew more rapidly or abundantly on oil, sometimes becoming more floccose or forming a dense mycelial mat throughout the oil. In many cases, the appearance of the oil changed from glossy and somewhat fluid to leathery and firm. Colony diameter alone was not a useful character because some small colonies (e.g., some species of *Penicillium*) appeared to sequester oil into a leathery mass. Species of *Acremonium* had a tendency to grow quickly on the medium with oil, forming a very fine mycelium which spread beyond the oil zone; growth on mineral medium was less abundant and more localized. Oil did not adhere to the mycelium and the *Acremonium* isolates were designated as unable to utilize oil. Some cultures, such as *Rhizopus stolonifer*, grew relatively poorly on the crude oil and growth was restricted to the region of the plate covered by oil. Cultures of *Rhizopus stolonifer* grown in the absence of oil showed similar or slightly less mycelial growth and growth was not as localized, but spread out across the plate. When growth was restricted to the region of the plate covered by oil, isolates were scored as being able to grow on crude oil and were further tested for their ability to degrade crude oil using analytical methods.

BIODEGRADATION POTENTIAL OF FUNGAL ISOLATES

Isolates that demonstrated an ability to grow on NWC oil (Table 2.1) were analyzed by GC for their ability to degrade a suite of hydrocarbons in oil. Although growth alone may be useful in determining if an isolate can utilize hydrocarbons as a substrate, it does not indicate which specific hydrocarbons are utilized unless the growth substrate is a pure compound. Isolates of a few species that did not show significant

growth on crude oil were included because of their prevalence at particular sites or previous reports in literature of their ability to utilize hydrocarbons. These were *Acremonium butyri, Acremonium kiliense, Acremonium strictum,* an unidentified species of *Acremonium, Aspergillus niger, Geomyces pannorus, Gliocladium* cf. *catenulatum,* an unidentified species of *Gliomastix* and *Paecilomyces variotii.*

GC analysis confirmed that all of the species demonstrating growth on crude oil altered the chemical composition of the oil, with the exception of *Penicillium* cf. *variabile, Rhizopus stolonifer* and *Ulocladium consortiale.* Growth of these cultures on medium with oil appeared to be slightly greater than on medium lacking oil, and thus they were initially designated as capable of growing on oil (Table 2.1). However, GC analyis confirmed that these cultures did not alter the chemical composition of GC-resolvable oil components. The ability of all other cultures to grow on crude oil was verified by comparing GC profiles of unfractionated oil from isolates following a 14 day incubation (Table 2.1; see below and Fig. 2.3 for comparison).

Residual oil did not readily separate from mycelium in cultures that achieved good to significant levels of biodegradation. Physical abrasion of the mycelium with a glass rod was required to enhance recovery of mycelium-associated oil. Where isolates did not achieve biodegradation of the oil, residual oil was readily recovered with pentane and did not require physical abrasion.

The GC profiles, shown in Figure 2.3, demonstrate the various levels of biodegradation of NWC oil (incubation at ≈ 22 °C). In most cases, isolates preferentially attacked the saturate fraction, varying from slight degradation of *n*-alkanes (Fig. 2.3C) to almost complete degradation of the saturate fraction (Fig. 2.3E) in comparison with the

sterile control (Fig. 2.3A). Where GC profiles of residual oil resembled profiles of sterile controls (Fig. 2.3B), it was assumed that no degradation took place. Overall, *Penicillium* species demonstrated the greatest degree of hydrocarbon biodegradation, removing almost all the *n*-alkanes (*n*-C_{12} - *n*-C_{26} range) and significantly altering the isoprenoids pristane and phytane. The profile of *Penicillium* cf. *isariiforme* (D-DV1-3-1B; Fig. 2.3E) exemplifies this pattern in the genus. In general, the majority of isolates that grew on crude oil had GC profiles with the *n*-alkanes almost or completely degraded, but some branched and cyclic alkanes and isoprenoids were left relatively unaltered (Fig. 2.3D). Isolates of *Rhizopus stolonifer* and *Aspergillus niger*, two species previously reported in literature to degrade oil, did not alter NWC oil (Fig. 2.3B). Isolates of *P.* cf. *variabile* also did not achieve any discernible degradation of crude oil despite it being one of the most frequently isolated species from KS.

GC profiles of the aliphatic fraction of crude oil (not shown) resembled profiles of unfractionated residual oil for all levels of degradation (Fig. 2.3A-E). The similarity of the profiles can be attributed to the predominance of the alkanes relative to other fractions in the unfractionated oil, therefore results of aliphatic degradation can be readily observed on unfractionated oil GC profiles. No species were observed to significantly degrade the aromatic fraction of NWC oil. However, *Aspergillus* sp. 2 (D-WG1-4-1G) slightly altered some of the low-molecular-weight aromatics (Fig 2.4B); the GC profile of the aromatic fraction of NWC oil after incubation with *Aspergillus fumigatus* (S-WG1-4-1C) was similar. The amount of residual oil recovered from cultures was slightly less than residual oil from the sterile controls and was presumed to be the result of oil adhering to the mycelium rather than degradation. All other GC profiles of the aromatic fraction

from NWC oil incubated with fungi resembled the aromatic profile of the sterile control (Fig. 2.4A).

Few isolates tested for degradation at low temperatures (5°C) during a 56 day incubation period on crude oil showed significant effects. GC profiles showed that the isolates *Oidiodendron griseum* (S-BL2-4-2) and *Penicillium thomii* (D-WG1-1-1) slightly degraded the *n*-alkanes *n*-C_{12} - *n*-C_{22} of the saturate fraction (2.5B). An unidentified *Penicillium* species significantly degraded the *n*-alkanes (*n*-C_{12} - *n*-C_{26} range) but left the isoprenoids pristane and phytane relatively undegraded (Fig. 2.5C).

RADIORESPIROMETRY

A group of isolates (Table 2.3) was selected for further examination of their ability to degrade hydrocarbons using NWC oil spiked with *n*-[1-^{14}C]-hexadecane as a growth substrate. Mineralization of *n*-[1-^{14}C]-hexadecane is summarized in Table 2.3. The isolates *Aspergillus fumigatus* (D-WG1-4-1; S-WG1-4-1), *Neosartorya fischeri* (S-NW1-1-2), *Oiodiodendron griseum* (S-BL2-2-2; S-BL2-3-1), *Penicillium thomii* (D-WG1-1-1), *Phialophora americana* (S-NW1-6-1) and *Pseudallescheria boydii* (D-NW2-7-2) exhibited the greatest amount of mineralization, producing more than 10% $^{14}CO_2$ from *n*-[1-^{14}C]-hexadecane. The isolates *Aspergillus niger* (S-NW1-7-1), *Geomyces pannorus* (D-BL1-6-1), *Paecilomyces variotii* (D-DV1-2-2), *Rhizopus stolonifer* (S-BL2-1-2) and *Ulocladium consortiale* (D-BL1-3-1) showed minimal or no *n*-[1-^{14}C]-hexadecane mineralization, ranging from values of 0.5 - 1.5%. None of the *Fusarium* species demonstrated significant mineralization of *n*-[1-^{14}C]-hexadecane, which contrasts with GC results of the same isolates grown on agar medium (Table 2.1).

Generally, recovery of $^{14}CO_2$ correlated well with loss of the compound *n*-[1-^{14}C]-hexadecane in residual oil (Table 2.3). An exception was *Neosartorya fischeri* (S-NW1-1-2) which produced little more than 5% $^{14}CO_2$, yet only 24% of *n*-[1-^{14}C]-hexadecane was recovered. This was the second lowest percent recovery of *n*-[1-^{14}C]-hexadecane; only 22.3% *n*-[1-^{14}C]-hexadecane was recovered from the culture of *Aspergillus fumigatus* (D-WG1-4-1), but more than 34% was mineralized to $^{14}CO_2$. Figure 2.6 is an illustration of Table 2.3 and shows the correlation of decreasing recovery of residual *n*-[1-^{14}C]-hexadecane with increasing production of $^{14}CO_2$.

For cultures inoculated as mycelial agar plugs into liquid medium (see methods), in addition to CO_2 recovery, the remaining culture medium was separated into three fractions (Fig. 2.2): pentane-soluble (containing parent compound), aqueous (polar metabolites), and biomass. The biomass fraction was rinsed with CH_2Cl_2 to remove superficial oil or metabolites adhering to the hyphae. Mineralization also was correlated with recovery of ^{14}C in the aqueous fraction; as more $^{14}CO_2$ was produced, more ^{14}C was recovered in the aqueous fraction, likely as polar metabolites such as alcohols or fatty acids (Fig. 2.6). There was no correlation between $^{14}CO_2$ or ^{14}C in the pentane fraction and the amount recovered in the biomass fraction or the CH_2Cl_2 fraction.

Most cultures tested for their mineralization ability showed a visible increase in the amount of mycelium present after the two week incubation. However, amount of growth usually correlated with the amount of mineralization. Significant growth was correlated with high levels of mineralization (> 5%) and was demonstrated by *Aspergillus fumigatus* (S-WG1-4-1; D-WG1-4-1), *Neosartorya fischeri* (S-NW1-1-2), *Oiodiodendron griseum* (S-BL2-2-2; S-BL2-4-2; S-BL2-3-1), *Penicillium thomii* (D-WG1-1-1),

Phialophora americana (S-NW1-6-1) and *Pseudallescheria boydii* (D-NW2-7-2). Mycelium formed as balls or pellets or occasionally remained as loose wefts sloughing off the agar plug. In other cultures, such as *Aspergillus niger* (S-NW1-7-1), *Fusarium avenaceum* (S-WG1-2-1) and *Rhizopus stolonifer* (S-BL2-1-2), low growth was correlated with low mineralization (< 5%), even when some exogenous carbon was present in the inoculum plug. One exception was *Penicillium thomii* (D-WG1-1-1), which showed abundant growth yet only mineralized 3.7% of *n*-[1-^{14}C]-hexadecane.

Isolates tested for their ability to mineralize *n*-[1-^{14}C]-hexadecane in liquid culture were also analyzed by GC. A parallel experiment using non-labeled oil in liquid medium was performed and, following a two week incubation, residual crude oil was recovered and analyzed. Isolates that degraded crude oil on agar media did not necessarily degrade it equally in liquid medium. For example, *Pseudallescheria boydii* (D-NW2-7-2) showed similar GC profiles following incubation on liquid media or agar media (Fig. 2.7A&B), but many other isolates showed significantly less ability to degrade oil in liquid. The GC profile of residual oil recovered from liquid cultures of *Aspergillus fumigatus* demonstrated significantly less degradation than a parallel culture on agar medium (Fig. 2.7C&D). Similar results were obtained with *Oidiodendron griseum* (S-BL2-2-2) and *Phialophora americana* (S-NW1-6-1). *Neosartorya fischeri* (S-NW1-1-2) was an exception: almost no free residual oil was recovered from this culture, although it appeared that residual oil was trapped within a significant amount of mycelial growth (Fig. 2.8E). Some free residual oil still remained in *Neosartorya fischeri* cultures incubated with *n*-[1-^{14}C]-hexadecane-spiked crude, but it was visibly evident that there was oil trapped within the mycelium. More than 20% percent ^{14}C was recovered in the

biomass and CH_2Cl_2 fractions combined. GC profiles of residual oil incubated with *Fusarium* species in liquid cultures did not show that a reduction in peak height of compounds had occurred.

None of the isolates was able to mineralize [9-^{14}C]-phenanthrene to $^{14}CO_2$ (Table 2.8; Fig. 2.8). Amount of $^{14}CO_2$ recovered was similar to the control value of 0.1%. Although more ^{14}C was recovered in the aqueous phase of all cultures compared to the sterile control, the difference was not significant; 5.3% ^{14}C was recovered from the control and 8.6% from *Fusarium avenaceum* (S-WG1-2-1), the highest amount from any culture. Similar to the *n*-[1-^{14}C]-hexadecane results, there was no correlation between [9-^{14}C]-phenanthrene recovered in the pentane fraction and ^{14}C recovered in the cell or CH_2Cl_2 fraction. However, 15% or more ^{14}C was recovered from the combined biomass and CH_2Cl_2 fractions in *Aspergillus fumigatus* (D-WG1-4-1C), *Neosartorya fischeri* (S-NW1-1-2) and *Pseudallescheria boydii* (D-NW2-7-2) cultures.

DISCUSSION

Many species with the ability to degrade hydrocarbons were isolated from petroleum-contaminated soil (Table 2.1). Most were hyphomycetes with an affinity to teleomorphic genera belonging to at least six families in five different orders within the division Ascomycota (Table 2.5). This indicates that the ability to degrade hydrocarbons may have originated early in fungal evolution or as a result of convergent evolution. Nyns et al. (1969) looked at the ability to degrade hydrocarbons as a taxonomic character but concluded that hydrocarbon degradation is strain-specific and not necessarily a

property of any given species or genus. Although teleomorphic genera of hyphomycetes observed to degrade hydrocarbons are related to different families and even different orders, the ability to degrade hydrocarbons appears to be a trait common to those genera. It does not appear to be random and restricted to one or a few species, but is exhibited by several species within certain families. Isolates in this study observed to degrade hydrocarbons are found mostly within two families, the Hyprocreaceae and the Trichocomaceae. The hyphomycete genera with teleomorphic affinities to these families include: *Aspergillus*, *Fusarium*, *Paecilomyces* and *Penicillium* (Hawksworth et al. 1995; Table 2.5). Another genus with species commonly reported to degrade hydrocarbons is *Trichoderma* (Chapter 1, Table 1.1) which also has affinities to the Hyprocreaceae (Hawksworth et al. 1995).

Pseudallescheria boydii, along with its anamorphs *Graphium* and *Scedosporium*, was observed to degrade hydrocarbons and belongs to the family Microascaceae in the order Microascales. Although this species may be unique in this genus or family regarding the ability to degrade hydrocarbons, there have been reports of other species labelled as "*Graphium*" with the ability to degrade hydrocarbons (Davies et al. 1973; Llanos and Kjoller 1976; Snellman et al. 1988; Zajic et al. 1969). Other *Graphium* species reported include *G. fructicolum* (Llanos and Kjoller 1976), *G. rubrum* (Lowery et al. 1968) and *G. putredinis* (Oudot et al. 1993), none of which have been connected to a teleomorphic genus. However, it is possible that these species are related to *Pseudallescheria* or other genera in the Microascaceae known to have *Graphium* anamorphs (i.e., *Kernia* and *Petriella*). The ability to degrade hydrocarbons in this family may be more widespread than currently believed.

Similarly, *Oidiodendron griseum*, an anamorph affiliated with the family Myxothrichaceae (Hambleton et al. 1998), was observed to degrade hydrocarbons; Nyns et al. (1968) also observed growth of an *Oidiodendron* sp. on hydrocarbons. The isolation of species of *Oidiodendron* has not been reported previously from hydrocarbon-contaminated soil and their ability to utilize hydrocarbons has not been examined. However, similar to *Pseudallescheria boydii*, the ability to degrade hydrocarbons may be more widespread within the Myxothrichaceae, and not simply a unique characteristic of the species *Oidiodendron griseum* or the isolates observed in soils from BL.

Some of the most common species isolated in this study belong to the genera *Aspergillus*, *Fusarium* and *Penicillium*, with *Penicillium* species making up the majority. Species from these genera were among the most effective hydrocarbon degraders isolated. Also, *Pseudallescheria boydii* was common and isolated from all sites except KS. Some common species that did not utilize hydrocarbons and which were found at most sites include *Acremonium* spp., *Aspergillus niger* and *Gliocladium* cf. *catenulatum*. Species of *Trichoderma* were recovered on all isolation media from all sites, though their ability to degrade hydrocarbons was not tested for reasons outlined in the results.

The species isolated are more likely a reflection of the isolation methods and media used than a true representation of the fungal community found in hydrocarbon-contaminated flare pits, representing only a small subset of those species. The dilution method favoured appearance of species that produce small conidia in large numbers such as species of *Acremonium*, *Aspergillus*, *Gliocladium*, *Penicillium* and *Trichoderma*. This method may exaggerate the importance of these fungi in the soils they were isolated from while selectively excluding others (Dix and Webster 1995).

Species richness was greater on direct isolation plates (Table 2.1). Not only would spores be present from species that are typically heavy sporulators, but spores and vegetative propagules from fungi that do not rely as heavily on rapid sporulation for dispersal would also be present. However, one problem with using the direct isolation method, particularly on selective media, is that the soil provides a substrate for fungi to grow on. Thus, in instances where certain fungi may be sensitive to inhibitors such as cycloheximide or benomyl, fungi are able to avoid the inhibitory effects by obtaining carbon from sources within the soil and not the medium containing the inhibitors. Acquiring residual carbon from the soil may also explain the appearance of non-hydrocarbon degraders on OAS medium.

Several types of isolation media were chosen to isolate representative hydrocarbon-degrading species from the soil fungal community of hydrocarbon-contaminated soils and to ensure that one group of fungi did not predominate and skew the appearance of the fungal community in hydrocarbon-contaminated soils. However, despite including five isolation media and two isolation procedures, the resulting isolates are likely still a very small subset of the community that exists in the contaminated soils.

MEA+T medium (containing a carbon source readily utilized by most hyphomycetes) was used as a general isolation medium to allow for the growth of most species. However, it is biased toward fungi that are quick sporulators and spread rapidly. Often, species of fungi in close proximity will exhibit antagonistic behaviour towards each other, resulting in suppressed growth of one or both species. For example, *Trichoderma* species are known to produce a wide range of anti-fungal volatiles (Dix and Webster 1995). Alternatively, fungi may resort to a predominantly submerged growth

habit in the presence of other fungi, complicating the isolation of these fungi. It is generally impossible to differentiate hyphae of submerged fungi on the basis of hyphal characteristics. *Gliocladium*, *Penicillium* and *Trichoderma* species are examples that often masked and prevented the isolation of some species or inhibited germination and growth of others. Inhibitors, such as cycloheximide and benomyl, were used to reduce the numbers of these fast growing fungi to allow the germination and growth of others.

MEA+T+B contains benomyl which binds to tubulin subunits, consequently preventing the formation of mitotic spindles necessary for mitotic division (Kendrick 1985). This was used to inhibit growth of some of the rapidly spreading fungi that would dominate on MEA+T medium, allowing sporulation of slower growing fungi. Species of *Aspergillus*, *Beauveria*, *Phialophora*, and *Rhinocladiella* were isolated more frequently on this medium while numbers of *Penicillium* species were reduced. MYB medium containing cycloheximide, which binds to ribosomes and prevents protein synthesis (Kendrick 1985), was used for similar purposes as MEA+T+B medium (i.e., to suppress growth of certain fungi). Inhibition of species sensitive to cycloheximide would allow for the germination and growth of other species present in the soil. *Pseudallescheria boydii* was common on this medium as well as *Chaetomium globosum*, *Exophiala jeanselmei* and *Geomyces pannorus*. Growth of *Penicillium*, *Aspergillus* and *Trichoderma* spp. was generally suppressed.

CMA+T provides corn meal as a source of carbon and is not as readily assimilated as malt extract, thus allowing fungi capable of growth on this substrate the opportunity to proliferate. *Neosartorya fischeri*, *Oidiodendron griseum* and *Paecilomyces variotii* were

frequent isolates on this medium. *Gliocladium*, *Penicillium* and *Trichoderma* species often overgrew the plates, masking and limiting the successful isolation of others.

It is difficult to differentiate species of *Penicillium* and *Aspergillus* on MEA or other general media, but colonies on CYA medium are usually distinct. For future isolations of soil fungi, this may be one medium to consider. Considering that *Aspergillus* and *Penicillium* species are common soil isolates and commonly found in hydrocarbon-contaminated soil, the use of CYA may aid in isolation by differentiating colonies. Other species of *Aspergillus* and *Penicillium* may have escaped detection because of similarities on isolation media.

It is interesting to note that the majority of the *Penicillium* species isolated in this study belonged to the subgenus *Biverticillium,* whereas most of the *Penicillium* species observed by Launen et al. (1995), belonged to the subgenus *Furcatum*. One exception in this study is *Penicillium janthinellum*, which was one of the most frequent isolates and belongs to the subgenus *Furcatum*. It is difficult to know if the difference between studies was because of geography, contamination, or isolation technique and whether hydrocarbon-degrading species are more common to one subgenus or the other.

Using a medium for hydrocarbon-adapted fungi (HAF; Oudot et al. 1987) or an OAS (used in this study), is beneficial for isolating fungi that are able to utilize or degrade hydrocarbons. However, dormant spores of non-hydrocarbon degrading isolates may be transferred inadvertently and develop on secondary media, thus leaving the impression that they were capable of growth on a hydrocarbon substrate. Also, some non-hydrocarbon-degrading fungi may grow, utilizing metabolites produced by other organisms actually utilizing the hydrocarbon or other carbon sources present in the soil,

as was observed by Davies et al. (1973) when a *Trichoderma* species grew only in the presence of a *Graphium* sp. when incubated with pure hydrocarbons. Generally, this type of medium was effective at isolating fungi capable of utilizing or degrading hydrocarbons.

Using OAS medium or a hydrocarbon-substituted medium for isolating fungi from hydrocarbon-contaminated soil provides a range of hydrocarbons, but still may lack potential growth compounds. Davies and Westlake (1979) tested fungi on a variety of crude oils and observed that fungi exhibited different levels of mycelial growth and did not grow on some crude oils. Davies and Westlake (1979) attributed this to the absence of certain compounds in the oil required for growth. Snellman et al. (1988) came to similar conclusions following incubation of fungi on different oil types. Alternatively, certain compounds may be present in the oil that are toxic to fungi. Because the chemical composition of crude oils differs widely, an alternative approach would be to use several types of crude oil (as done by Davies and Westlake 1979) or perhaps using a crude oil or hydrocarbon similar to that present in the contaminated soil being studied.

The growth habit of organisms on crude oil may complicate determination of different species. A number of fungi isolated from this medium did not readily sporulate and visible vegetative hyphae were used for isolation. Because hyphal characteristics are not diagnostic, more fungi were transferred from isolation plates than necessary. Part of the problem in this study may have been the amount of oil added to the isolation medium: one millilitre was used per plate, whereas in the studies done by Oudot et al. (1987; 1993), 0.1 or 0.2 mL of oil was added. A relatively large volume of oil mixed within the agar (see methods) contributed to a soft texture of the substrate and formed pools on the

surface. Conidiophores would be unable to firmly anchor to the substrate, thus preventing an upright position or, from disturbance of moving plates for examination, conidiophores may have collapsed or toppled over. This phenomenon was observed with *Penicillium* species growing on mineral salts agar incubated with crude oil. In cultures that did not form a tough leathery surface with the oil, conidiophores that were upright toppled over and adhered to the surface of the oil upon movement of plates and removal of the Petri plate cover. Consequently, coniophores were not readily observable, complicating the isolation of fungi.

Few species with a predominantly submerged growth habit (e.g., *Aureobasidium pullulans*) were isolated. Profuse growth of *Gliocladium*, *Penicillium* and *Trichoderma* species made isolating fungi with submerged hyphae difficult, and consequently the best way to retrieve fungi with this growth pattern would be to macerate agar chunks and plate these out and then quickly separate the newly formed colonies. However, this can be extremely time consuming.

It was not surprising to see that the most common isolates were species of *Aspergillus* and *Penicillium*. TEH levels in the soils from the flare pits ranged from just under 5% to close to 20%, indicating that levels of hydrocarbon contamination are high. These hydrophobic materials limit the water availability and increase the matric potential. Often compounding this problem in flare pits are high salt concentrations which lower the osmotic potential. *Aspergillus* and *Penicillium* species, with affinities to the Eurotiales, are known osmotolerant (Dix and Webster 1995). Osmotolerant fungi are capable of growth where the osmotic potential is low (e.g., -50 MPa), caused by a high solute concentration. Particularly in soils in warmer climates that contain high salt

concentrations, *Aspergillus* species are common and able to grow at exceedingly low water activities (0.70; Dix and Webster 1995). *Pseudallescheria boydii* is also osmotolerant and has previously been observed to grow in environments with high salt concentrations (2% NaCl and 5% $MgCl_2$; de Hoog et al. 1994). It was a common isolate from all flare pits except KS.

In xeric conditions, both *Aspergillus* and *Penicillium* species are common, with *Aspergillus* species collectively the most xerotolerant group and capable of growth with water potentials as low as -40 Mpa (Dix and Webster 1995). *Penicillium* species tend to replace *Aspergillus* species in temperate climates, where the substrata tend to have a higher water content. The common occurrence of *Penicillium* species relative to species of *Aspergillus* was observed in this study, particularly at the three central sites. However, *Aspergillus* species are able to compete successfully with *Penicillium* species at higher temperatures or lower water activities (low matric potential) such as acitivities between 0.70 and 0.78 (Dix and Webster 1995). At activities higher (> 0.78), *Penicillium* species appear more successful. Growth of non-xerotolerant fungi occurs at activities of 0.85 and greater (Dix and Webster 1995). The KS flare pit is situated in a region where dry and hot conditions are common which may explain the increase in the number of *Aspergillus* species at this site as well as their frequent isolation. *Penicillium* species were also common at the KS site, with few other species being isolated.

Penicillium species were common at all sites, however the greatest species diversity was observed at DV, followed next by BL and WG. The least number of species was observed at KS and NW. Generally, forest soils support their own characteristic *Penicillium* populations and carry a greater diversity of species compared to

grasslands (Dix and Webster 1995). BL, DV and WG flare pit sites were all within close proximity to forests, whereas KS was amidst grassland. The number of *Penicillium* species isolated from NW may be fewer because of lower annual temperatures.

Acremonium species and *Gliocladium* cf. *catenulatum* were frequently isolated from most sites, but were unable to degrade hydrocarbons or grow on crude oil as the sole carbon source. They may use metabolites released by hydrocarbon-degrading fungi such as alcohols, aldehydes and fatty acids. For example, Zajic et al. (1969) isolated a strain of *Trichoderma viride* along with a *Graphium* sp. from sewage sludge. The *Graphium* sp. was able to utilize gaseous hydrocarbons as a sole carbon source, but the *Trichoderma* species was not. However, when *Trichoderma viride* was incubated with gaseous *n*-alkanes in the presence of the *Graphium* sp., it grew quite well, indicating that it was utilizing products released by the *Graphium* sp. during hydrocarbon metabolism.

The low numbers of zygomycetes (*Rhizopus stolonifer* from KS and BL) was surprising considering the number of species from the Mucorales that have been reported to grow on or degrade hydrocarbons (Table 1.1, Chapter 1). Launen et al. (1995) reported zygomycetes only from soils that contained less than 3 µg/g soil of PAHs. This may indicate that the soils sampled in this study were too heavily contaminated to permit growth of zygomycetes. Although PAH levels were not available for most of the soils in this study, levels at the BL site ranged between 19.68 and 68.67 µg/g soil and no zygomycetes were isolated. If PAH levels at the other sites were similar to the levels measured at BL, this may explain the lack of isolates belonging to the zygomycetes. Media used specifically to suppress the growth of zygomyctes contain phenolics (such as

pentachlorophenol nitrobenzene), thus indicating that high levels of phenolics or aromatic compounds in the soil would have a similar effect.

Numerous species of *Aspergillus* and *Penicillium* (Table 2.1) were observed to degrade hydrocarbons as determined by GC analysis of residual crude oil. Isolates of *Aspergillus* cf. *tamarii* and *Aspergillus* cf. *niveus* were observed to degrade the *n*-alkanes in NWC oil. *A.* cf. *tamarii* was previously observed by Oudot et al. (1987) to degrade crude oil. *A.* cf. *niveus*, however, has not previously been reported in literature to degrade hydrocarbons. Most of the species isolated from all sites belonged to the genus *Penicillium*. Of these, *Penicillium* cf. *janthinellum*, *Penicillium pinophilum* and *Penicillium thomii* have previously been reported to degrade hydrocarbons (Chaineau et al. 1995; Kiehlmann et al. 1996; Launen et al. 1995; Oudot et al. 1987; Snellman et al. 1988). *P. janthinellum* was observed to degrade the saturate and aromatic fractions and slightly attack the resin fraction of crude oil (Oudot et al. 1987). Launen et al. (1995) and Kiehlmann et al. (1996) observed *P. janthinellum* transform pyrene and benzo[a]pyrene into monols, diols and quinones.

All species that were able to degrade oil were observed to degrade the aliphatics, particularly the *n*-alkanes (n-C_{12} - n-C_{26}). These compounds were likely used as a carbon and energy source, and not simply transformed into compounds less toxic to the fungi. Fungi generally oxidize aliphatics into primary or secondary alcohols, then to aldehydes or ketones, and then to monoic or short-chain fatty acids (Hoffman and Rehm 1976). Monoic acids can undergo β-oxidation forming C_2-units and short-chain fatty acids (Hoffman and Rehm 1976). The final product is carbon dioxide and water, resulting in complete mineralization of the hydrocarbons. Mineralization of these compounds to

carbon dioxide is supported by the results of isolates incubated with NWC oil spiked with *n*-[1-^{14}C]-hexadecane (Table 2.4; Fig. 2.6). Until recently, few studies of the degradative abilities of hyphomycetes (Kiehlmann et al. 1996, Launen et al. 1995, Sack et al. 1997 and Wunder et al. 1994) have employed the use of [^{14}C]-labelled compounds to determine if fungi are able to transform or mineralize hydrocarbons.

Significant mineralization (> 10%) of *n*-[1-^{14}C]-hexadecane was observed in cultures of *Aspergillus fumigatus* (D-WG1-4-1; S-WG1-4-1), *Neosartorya fischeri* (S-NW1-1-2), *Oidiodendrom griseum* (S-BL2-2-2; S-BL2-3-1), *Phialophora americana* (S-NW1-6-1) and *Pseudallescheria boydii* (D-NW2-7-2). *A. fumigatus* has been previously reported to degrade hydrocarbons (Chaineau et al. 1995; Oudot et al. 1993; Oudot et al. 1987; Snellman et al. 1988) as well as *P. thomii* (Oudot et al. 1987). *P. boydii* had been observed previously to degrade *n*-alkanes (Chapter 3), but this is the first confirmation of *n*-alkane mineralization. This is the first report of *N. fischeri*, *O. griseum* and *P. americana* to degrade hydrocarbons; *Phialophora jeanselmei* was previously reported by Davies et al. (1973) to grow on hydrocarbons.

Strains of the same species appear to be variable in their ability to utilize or degrade hydrocarbons. GC results demonstrated that not all strains of a species degrade hydrocarbons to the same extent and/or at the same rate (Table 2.2). Strains varied in the extent to which they degraded hydrocarbons, though all strains of a species were able to degrade hydrocarbons to some degree. Mineralization studies also supported these observations. Two isolates of *O. griseum* (S-BL2-2-2; S-BL2-3-1) demonstrated an ability to mineralize more than 25% of ^{14}C-hexadecane, yet a third isolate (S-BL2-4-2) yielded only slightly more than 5% $^{14}CO_2$. These abilities of an isolate to degrade

hydrocarbons varied during this study. For example, *P. thomii* was able to mineralize over 35% ^{14}C-hexadecane, but the second time the same isolate was grown under similar conditions, it mineralized less than 4% (Table 2.3). In both cases, there was significant mycelial growth. It is possible that the region of the colony that the plug was taken from was not physiologically very active at the time, affecting its ability to utilize the crude oil. Cultures generally exhibit a lag phase during which the physiological state adjusts to the conditions before the cultures begin to grow (Brown 1988). Another possibility is that the colony may have colonized and utilized the carbon from the MEA plug prior to utilizing ^{14}C-hexadecane as a substrate, malt extract being the preferred carbon source. If the culture had been incubated for a longer period, degradation patterns may have been more congruent.

Aspergillus niger was frequently isolated from all sites except KS, but all isolates were unable to degrade crude oil. *A. niger* has been reported previously by Cerniglia (1984), Cerniglia et al. (1978), Chaineau et al. (1995), Oudot et al. (1987), Sack et al. (1997), Snellman et al. (1988) and Wunder et al. (1994) to grow on or degrade hydrocarbons.

None of the isolates tested was able to mineralize phenanthrene, nor did any demonstrate significant levels of transformation as determined by GC analysis. The results of phenanthrene mineralization were not surprising; PAHs are generally not used by fungi for carbon and energy sources, but may be cometabolized to hydroxylated products such as *trans*-dihydrodiols (Bezalel et al. 1996).

Although mineralization of PAHs is desirable because a number of them are carcinogenic (International Agency for Research on Cancer 1983), oxidation to another

form often reduces their mutagenic and carcinogenic nature. Oxidized products include *trans*-dihydrodiols, dihydrodiol epoxides, tetrahydrotetraols, phenols, quinones and sulfate, glucoronide, and glucoside conjugates (Cerniglia et al. 1985). Some of the metabolites can be more mutagenic (*trans*-dihydrodiols, dihydrodiol epoxides, and tetrahydrotetraols), but also may be more amenable to further oxidation and subsequent mineralization than the parent PAH (Launen et al. 1995). Transformation to PAH conjugates may be an important reaction in the decontamination and detoxification of PAHs, leading to the induction of biological effects (Cerniglia 1981; Cerniglia et al. 1989). PAH metabolites are generally more polar and therefore more water-soluble and more susceptible to enzymatic attack.

Phenanthrene was used a model of PAH biodegradation because it is often found in high concentrations in PAH-contaminated environments and because many PAHs containing a phenanthrene moiety are carcinogenic (Bezalel et al. 1996). Results indicated that little [9-^{14}C]-phenanthrene transformation occurred (Table 2.5, Fig. 2.8). Relatively low percentages of ^{14}C were recovered in the aqueous fractions and a high percentage was recovered in the pentane fraction, likely as residual parent compound. Although the mean ^{14}C recovery was as high as 8.6% (range of 6.2 - 11.2%) in the aqueous fraction of *Fusarium avenaceum*, 5.3% was recovered in the aqueous fraction of the parallel sterile control; all isolates tested had ranges within one standard deviation of the sterile control. However, Wunder et al. (1994) reported that most of the metabolites they recovered were from the mycelial extract and very little was from the aqueous fraction.

Although the isolates tested were not expected to mineralize [9-^{14}C]-phenanthrene, higher levels of ^{14}C recovered in the aqueous or CH_2Cl_2 fraction were expected, particularly by *Aspergillus fumigatus* and *Oidiodendron griseum,* in the form of polar metabolites. Isolates of both species were able to mineralize *n*-[1-^{14}C]-hexadecane, and thus had a carbon and energy source present for growth, but they were unable to gratuitously degrade the PAH as part of their normal metabolic process. Haselwandter et al. (1990) observed *O. griseum* to mineralize ^{14}C-labeled lignin and a dehydropolymer of lignin. With the ability to degrade structural analogues, *O. griseum* was expected to degrade phenanthrene, although GC analysis did not show any transformation of the aromatic fraction. *A. fumigatus* was previously observed to degrade compounds in the aromatic fraction, transforming nearly 30% of the fraction (Oudot et al. 1993).

Both *Pseudallescheria boydii* and *Neosartorya fischeri* had mean ^{14}C recovery values of over 12% in the biomass. Although this may represent cell-associated metabolites, it is more likely residual parent compound associated with the mycelium. Kiehlman et al. (1996) recovered residual ^{14}C from cultures of *P. janthinellum* incubated with chrysene and found more than 41% of ^{14}C in the cellular fraction. Following ethyl acetate extraction and repeated rinses of the cells with CH_2Cl_2, 33% was determined to be the unaltered labeled chrysene while only 8% was incorporated into metabolites. This indicates that significant amounts of hydrocarbons are sequestered by the mycelium, perhaps as a means of detoxification. Over time, sequestered compounds may be transformed into oxidized products, or remain tied up in the mycelium.

Neosartorya fischeri did not show particularly high levels of *n*-[1-^{14}C]-hexadecane mineralization and did not degrade [9-^{14}C]-phenanthrene. This species did

incorporate a large quantity of the oil into the mycelial mass (not into the cells). In one experiment, after incubation of *N. fischeri* with unlabeled NWC oil, almost no residual oil was recovered (Fig. 2.7). Although oil was not extracted readily from the cellular mass, it was visibly trapped within the mycelium and did not appear to be taken into the cells. It is not cleat if this initial binding phase leads to enzymatic activity which may degrade the oil. Oil binding may also account for low values of total ^{14}C recovered. Observations obtained from both isolates of *A. fumigatus* tested for [9-^{14}C]-phenanthrene mineralization, where less than 73% of the labeled compound was recovered, indicate that the labeled compound was sequestered by the mycelium apparently as a method of detoxification. Similarly, Kiehlmann et al. (1996) recovered over 95% of the radiolabel from the control flask, while only recovered approximately 64% from the flask inoculated with *Penicillium janthinellum*, thus indicating that this isolate also sequestered the oil in the mycelium.

There are a number of possible explanations for the lack of degradation of aromatic compounds. The first, supported by the results of GC analysis, is that none of the isolates is capable of degrading aromatic compounds, at least with hydrocarbons as the sole carbon source. The second is that most of the isolates do not perform as well in liquid culture as on agar medium. Figure 2.7 shows that some of the isolates transformed much less NWC oil in liquid culture than on agar medium. High water activity of the liquid medium may reduce the ability to take up compounds or may disrupt regular physiological activities. Most of these fungi are isolated from conditions with low osmotic or matric potentials. The physiology of these organsims when immersed in a liquid may not include the production of the enzymes necessary to attack the aromatic

compounds or may cause an extended physiological lag time. Thirdly, culture agitation (by rotary shaking) may contribute to physiological stress. Most of the isolates grew as fungal balls rather than a mycelial mat which would more closely resemble their normal growth habit in soil. Hyphae in liquid may be prevented from growing out over the surface of the oil. Because oxidation of PAHs in filamentous fungi is metabolized intracellularly by the cytochrome P-450 monooxygenase system, preventing the uptake of oil into the cells would block the metabolism of PAHs. Davies and Westlake (1979) and Oudot et al. (1987; 1993) grew cultures under static conditions, thus allowing them a more natural growth environment and both studies observed degradation of aromatic compounds. In contrast, Kiehlman et al. (1996) and Launen et al. (1995) observed transformation of PAHs while cultures were grown in liquid medium on a rotary shaker at 240 rpm.

All cultures had been stored on MEA medium for at least six months prior to inoculation into liquid medium and incubation with hydrocarbons. This point leads to a forrth possible explanation: cultures incubated for extended periods of time on media that contain readily utilizable carbon substrates may lose the ability to degrade hydrocarbons. Isolates may need a certain stimulus or environmental factor to induce the necessary enzymes for metabolism by the P-450 monooxygenase system. When incubated on carbon sources that are readily metabolized, there is no need to produce enzymes capable of oxidizing complex substrates. Isolates such as *Fusarium avenaceum* (S-WG1-4-1) and *Fusarium subglutinans* (D-DV1-2-1), following a twelve month incubation period on MEA medium, showed markedly lower abilities to degrade crude oil than when originally tested by GC analysis. Launen et al. (1995) made similar observations with a *Penicillium*

sp. they had earlier observed to degrade benzo[a]pyrene. However, other isolates did not show a change in degradative ability following an extended period without exposure to hydrocarbons, including *Aspergillus fumigatus* (D-WG1-4-1), *Aspergillus* sp. 2 (D-WG1-4-1G), a number of *Penicillium* species and *Pseudallescheria boydii* (D-NW2-7-2), when re-tested by GC analysis for their ability to degrade hydrocarbons.

A final explanation for the lack of detectable degradation of PAHs is that the incubation period was too short. Oudot et al. (1987; 1993) incubated cultures for thirty days before completing chemical analyses and noting degradation of the aromatic fraction by isolates. Transformation of aromatics may be delayed, following the utilization of compounds that can be readily metabolized such as short to medium length aliphatics (*n*-C_{12} - *n*-C_{26} range). In contrast, Kiehlman et al. (1996) and Launen et al. (1995) observed degradation of chrysene, pyrene and benzo[a]pyrene after 10 days incubation. Both studies included dextrose as a carbon source. Wunder et al. (1994) observed a strain of *Aspergillus niger* oxidize pyrene; it was not until after the glucose in the medium had been completely metabolized that pyrene metabolites were produced. In this study, because there was a relatively large supply of aliphatics that the isolates were able to utilize, PAH-degrading enzymes may not have been induced, and upon exhaustion of the aliphatics, PAH degradation may eventually have occurred.

Although research over the last ten years has focussed on basidiomycetes and their ability to oxidize PAHs under laboratory conditions, considering hyphomycetes for *in situ* bioremediation of petroleum-contaminated soils has a number of advantages over basidiomycetes. First, hyphomycetes comprise the majority of fungal species present in the soil community. Second, hyphomycetes have the ability to degrade PAHs under

laboratory conditions (Kiehlmann et al. 1996; Launen et al. 1995; Oudot et al. 1987, 1993), thus they do possess the potential, although further research is required to determine their ability *in situ*. Lastly, hyphomycetes such as species of *Aspergillus*, *Fusarium*, and *Penicillium* are osmo- and xerotolerant, and would be able to survive and grow in a hydrophobic soil environment such as that contaminated with hydrocarbons. Basidiomycetes are extremely sensitive to changes in water potential. If water content is too high, aeration becomes a problem (although likely already a problem in petroleum-saturated soils) and if water content is too low (< -7.0 MPa), this is often the limiting factor restricting growth and slowing enzymatic activity (Dix and Webster 1995).

The species isolated in this study are likely a small subset of the actual fungal community present in hydrocarbon-contaminated soils. Considering that a large portion of the isolates in this study were capable of degrading hydrocarbons, there is likely a large number of indigenous soil fungi that were not isolated with the ability to degrade hydrocarbons. This ability appears relatively widespread among filamentous fungi, encompassing at least five families across four orders within the Ascomycota.

Although species in this study demonstrated degradation of hydrocarbons that was restricted to the aliphatic fraction, other studies (Kiehlmann et al. 1996; Launen et al. 1995; Oudot et al. 1987, 1993; Sack et al. 1997; Wunder et al. 1994) have demonstrated that filamentous fungi do possess the ability to oxidize aromatic compounds. Fungi generally degrade PAHs at rates slower and are less efficient than bacteria; however they do have the ability to hydroxylate a wide variety of compounds and have an advantage over bacteria with the ability to grow through the solid soil matrix (Cerniglia 1997).

Filamentous fungi therefore may play an integral role in the *in situ* biodegradation of pollutants in soils.

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Figure 2.1. Map of western and northern Canada showing location of hydrocarbon-contaminated sampling sites. 1, Norman Wells (NW); 2, Boundary Lake (BL); 3, Drayton Valley (DV); 4, Willesden Green (WG); 5, Kindersley (KS).

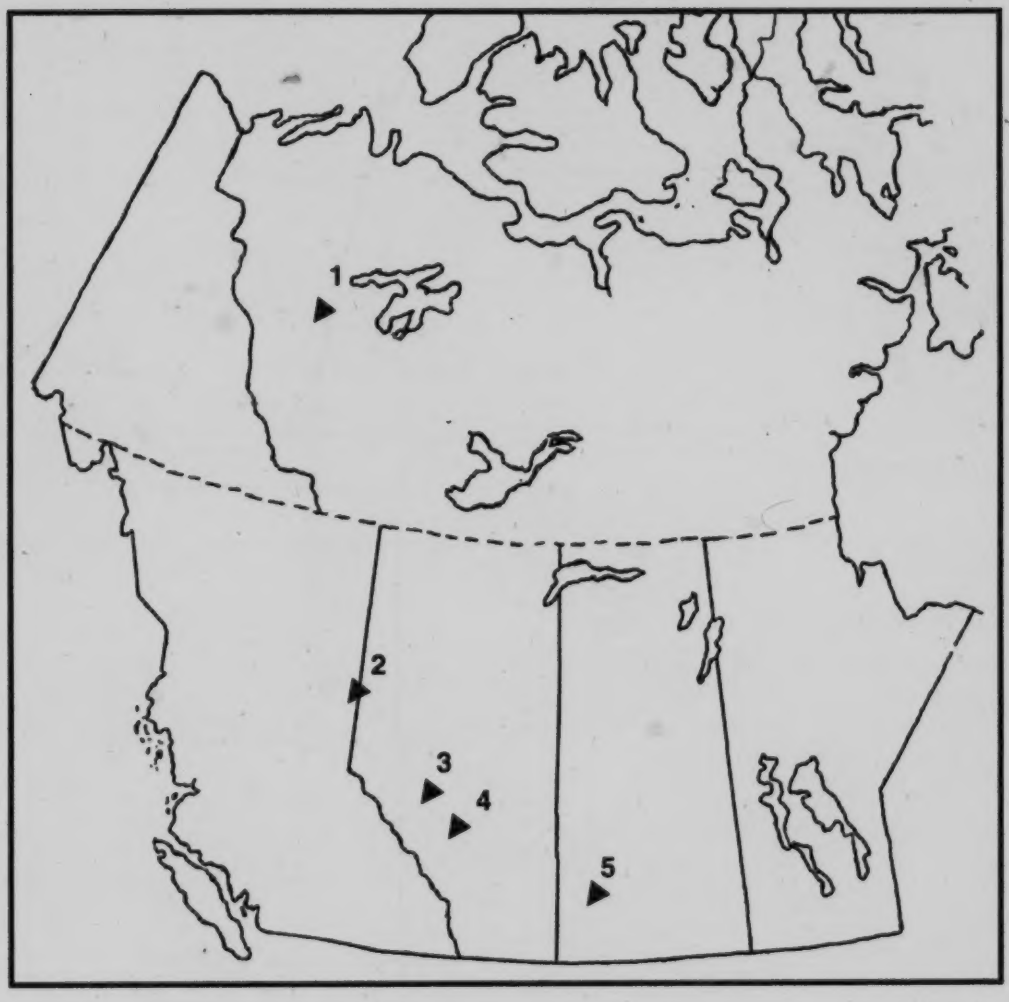
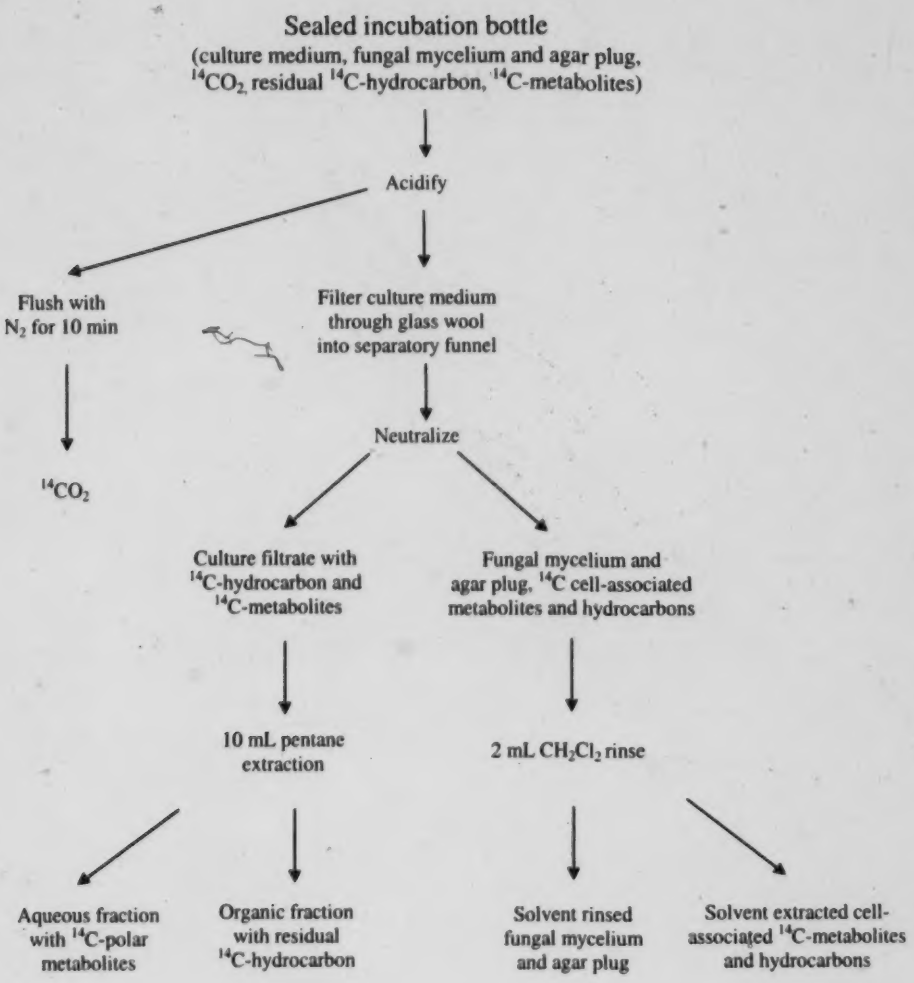

1
2
3
4
5

Figure 2.2. Method of recovery of ^{14}C in aqueous, organic and cell fractions following 14 day incubation of fungi in liquid culture with a growth substrate of NWC oil spiked with ^{14}C-hydrocarbon (hexadecane or phenanthrene).


Sealed incubation bottle
(culture medium, fungal mycelium and agar plug,
$^{14}CO_2$, residual ^{14}C-hydrocarbon, ^{14}C-metabolites)
Acidify
Flush with
N_2 for 10 min
Filter culture medium
through glass wool
into separatory funnel
Neutralize
$^{14}CO_2$
Culture filtrate with
^{14}C-hydrocarbon and
^{14}C-metabolites
Fungal mycelium and
agar plug, ^{14}C cell-associated
metabolites and hydrocarbons
10 mL pentane
extraction
2 mL CH_2Cl_2 rinse
Aqueous fraction
with ^{14}C-polar
metabolites
Organic fraction
with residual
^{14}C-hydrocarbon
Solvent rinsed
fungal mycelium
and agar plug
Solvent extracted cell-
associated ^{14}C-metabolites
and hydrocarbons

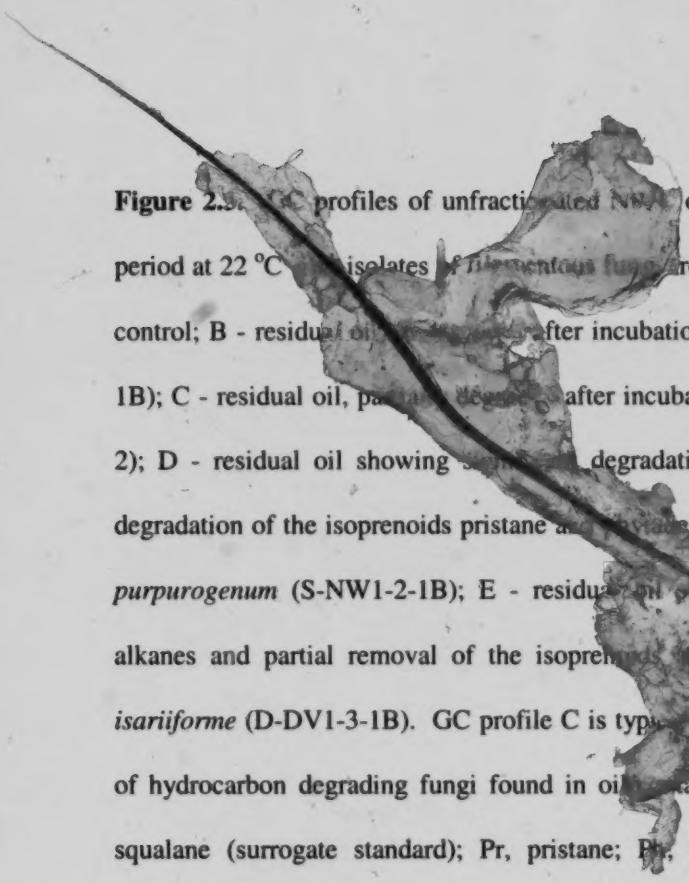


Figure 2.3. GC profiles of unfractionated NWA oil following a two week incubation period at 22 °C with isolates of filamentous fungi from oil-contaminated soils. A - sterile control; B - residual oil showing degradation after incubation with *Aspergillus niger* (S-NW1-7-1B); C - residual oil, partially degraded after incubation with *Fusarium* sp. 2 (D-BL1-3-2); D - residual oil showing degradation of *n*-alkanes and little or slight degradation of the isoprenoids pristane and phytane after incubation with *Penicillium* cf. *purpurogenum* (S-NW1-2-1B); E - residual oil showing complete degradation of *n*-alkanes and partial removal of the isoprenoids after incubation with *Penicillium* cf. *isariiforme* (D-DV1-3-1B). GC profile C is typical of crude oil incubated with majority of hydrocarbon degrading fungi found in oil contaminated soil in western Canada. S, squalane (surrogate standard); Pr, pristane; Ph, phytane; *n*-C_{16}, hexadecane; *n*-C_{24}, tetracosane. Profiles of residual oil in Fig.2.3B, C, D & E correspond to the levels of degradation (N, P, G & S) demonstrated by fungal species listed in Table 2.2

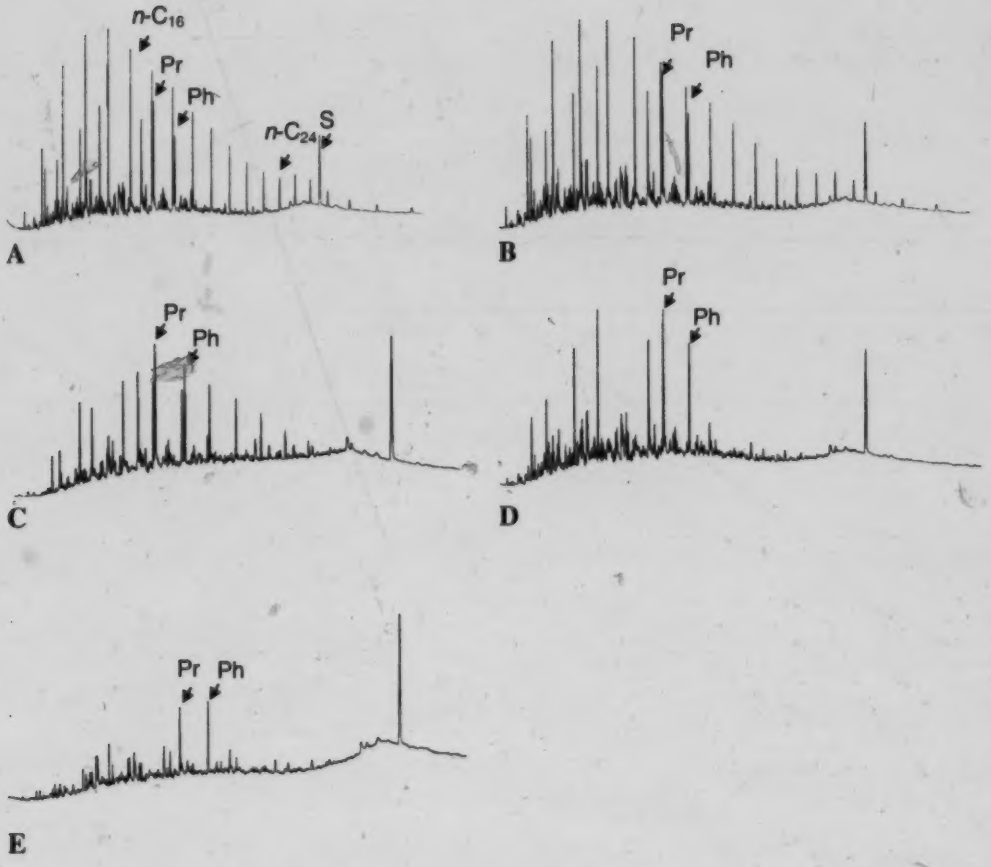
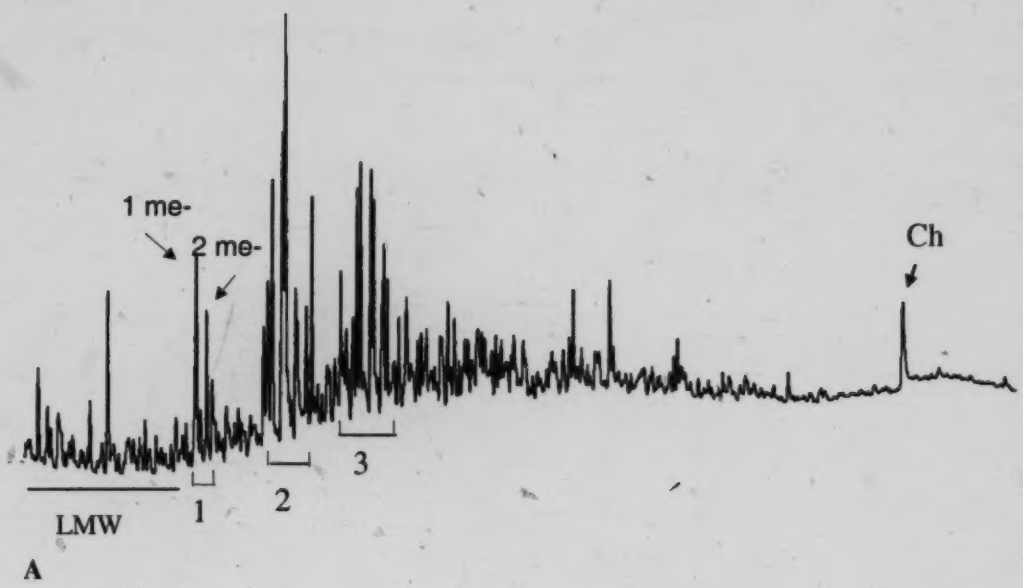
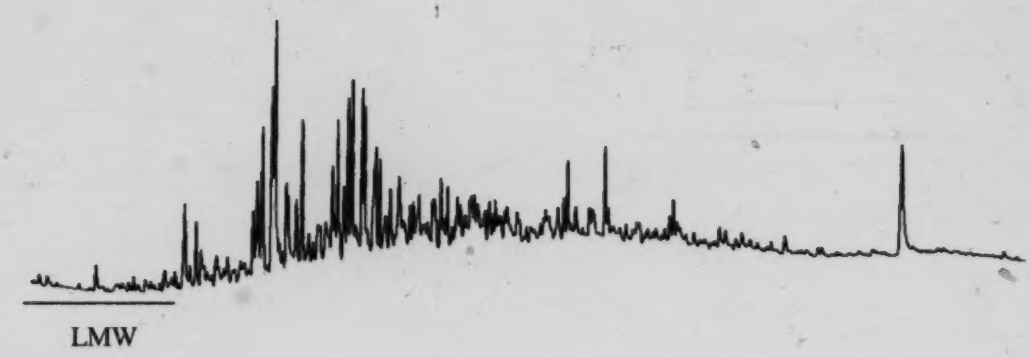

n-C16
Pr
Ph
n-C24
S
A
Pr
Ph
B
Pr
Ph
C
Pr
Ph
D
Pr
Ph
E

Figure 2.4. Gas chromatographic profiles of the aromatic fraction of Norman Wells Crude oil following a 14-day incubation with isolates of filamentous fungi. **A** - Sterile control, no hydrocarbon degradation observed; similar to profiles observed with most isolates; **B** - *Aspergillus* sp. 2 (D-WG1-4-1G), limited hydrocarbon degradation of low-molecular-weight aromatic compounds (LMW). Ch, chrysene (surrogate standard); 1, 1- and 2-methylnaphthalene; C_2-naphthalenes; 3, C_3-naphthalenes (Fedorak and Westlake 1981). Note: Not as much oil was recovered from the *Aspergillus* sp. 2 culture compared with the sterile control (relative to surrogate standard); possibly due to adherence of oil to mycelium rather than degradation.



A



B

Figure 2.5. GC profiles of fungal cultures incubated with Norman Wells Crude oil at 5 °C for 56 days. **A** - sterile control; **B** - residual oil showing degradation of *n*-C_{12} - *n*-C_{20} after incubation with *Oidiodendron griseum* (S-BL1-3-2), similar to the profile produced by *Penicillium thomii* (D-WG1-1-1) at the same temperature; **C** - residual oil demonstrating significant degradation of the saturate fraction, *n*-C_{12} and *n*-C_{26}, and little alteration of the isoprenoids pristane and phytane following incubation with an unidentified *Penicillium* species (S-NW1-2-1C). S, squalane (surrogate standard); Pr, pristane; Ph, phytane; *n*-C_{16}, hexadecane; *n*-C_{24}, tetracosane.

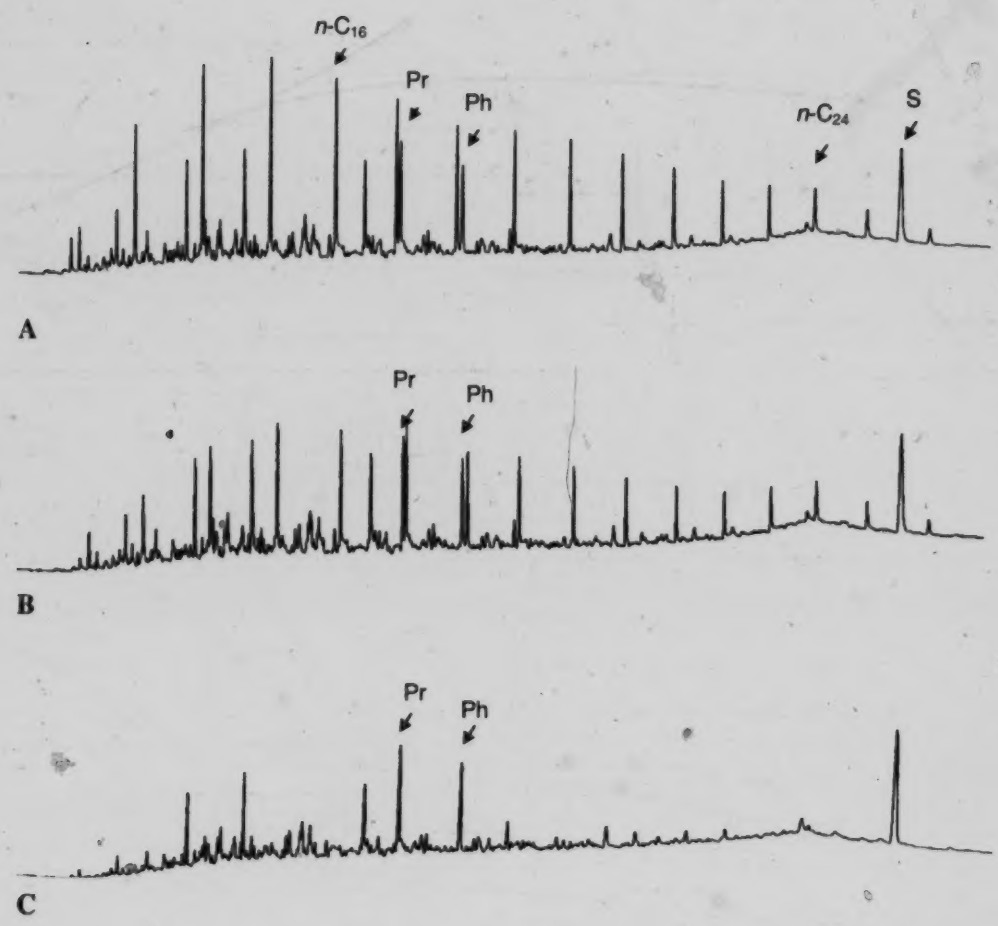

n-C16
Pr
Ph
n-C24
S
A
Pr
Ph
B
Pr
Ph
C

Figure 2.6. Percent ^{14}C recovered as $^{14}CO_2$ and in pentane, aqueous, CH_2Cl_2, and fungal cell biomass fractions following 14 days incubation of fungi with NWC oil spiked with *n*-[1-^{14}C]-hexadecane.

% 14C in pentane
% 14C in CH2Cl2
% 14C in biomass
% 14C in aqueous
% 14CO2
% 14C in fractions
0
20
40
60
80
100
120
Species
Ph. americana (S-NW1-6-1)
A. fumigatus (D-WG1-4-1)
O. griseum (S-BL2-2-2)
O. griseum (S-BL2-3-1)
P. boydii (D-NW2-7-2)
A. fumigatus (S-WG1-4-1)
N. fischerii (S-NW1-1-2)
O. griseum (S-BL2-4-2)
P. thomii (D-WG1-1-1)
F. subglutinans (D-DV1-2-1)
Fusarium sp. 2 (D-BL1-3-2)
F. avenaceum (S-WG1-2-1)
Fusarium sp. 1 (S-WG1-4-1)
Sterile control

Figure 2.7. Gas chromatographic profiles of Norman Wells Crude oil following a 14-day incubation with *Neosartorya fischeri* (S-NW1-1-2), *Penicillium thomii* (D-WG1-1-1) and *Pseudallescheria boydii* (D-NW2-7-2). **A** - oil incubated with *Penicillium thomii* in liquid culture, showing poor degradation of *n*-alkanes; **B** - oil incubated with *Penicillium thomii* on agar medium, showing significantly more degradation of *n*-alkanes; **C, D** - oil incubated with *Pseudallescheria boydii* in liquid culture and on agar medium, respectively, showing significant degradation of *n*-alkanes; **E** - almost no recovery of residual oil from liquid culture of *Neosartorya fischeri*; **F** - liquid culture sterile control. S, squalane (surrogate standard); Pr, pristane; Ph, phytane; *n*-C_{16}, hexadecane; *n*-C_{24}, tetracosane.

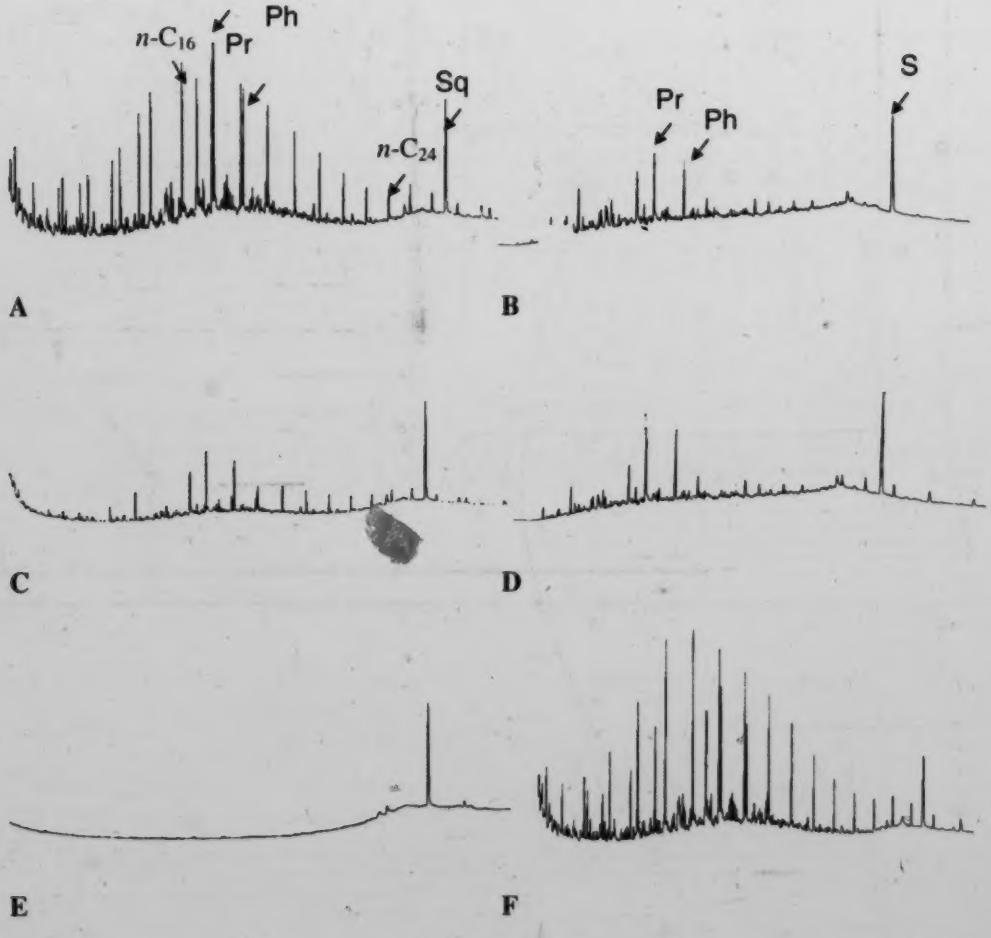

n-C16
Pr
Ph
Sq
n-C24
Pr
Ph
S
A
B
C
D
E
F

Figure 2.8. Percent ^{14}C recovered as $^{14}CO_2$ and in pentane, aqueous and CH_2Cl_2, and cell biomass fractions following 14 day incubation of fungi on NWC oil spiked with [9-^{14}C]-phenanthrene.

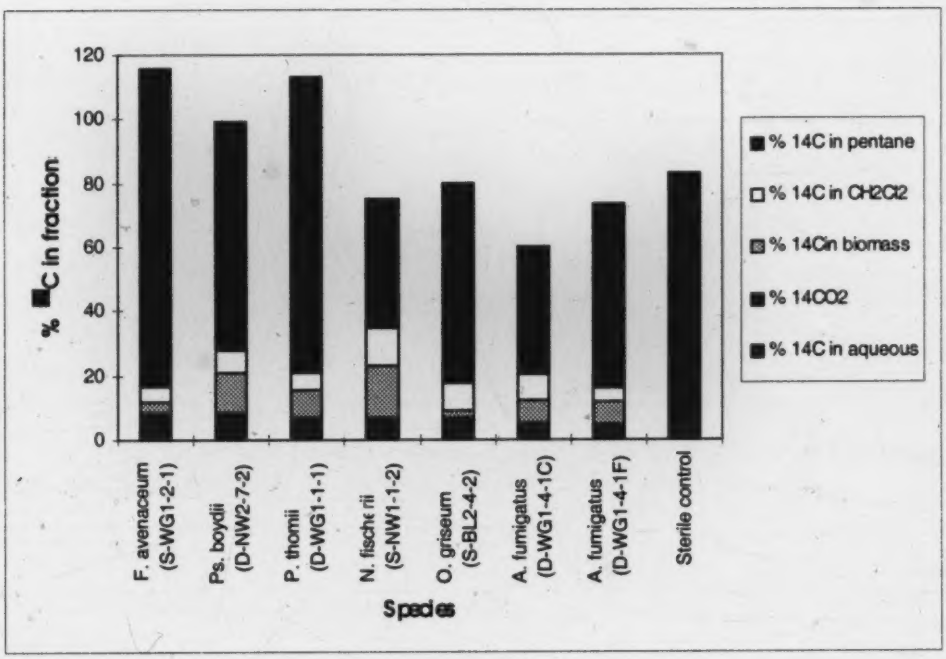

120
100
80
60
40
20
0
% ■C in fraction
% 14C in pentane
% 14C in CH2Cl2
% 14Cin biomass
% 14CO2
% 14C in aqueous
F. avenaceum (S-WG1-2-1)
Ps. boydii (D-NW2-7-2)
P. thomii (D-WG1-1-1)
N. fischeri (S-NW1-1-2)
O. griseum (S-BL2-4-2)
A. fumigatus (D-WG1-4-1C)
A. fumigatus (D-WG1-4-1F)
Sterile control
Species

Table 2.1. Species of filamentous fungi isolated from flare pits in western Canada. Also indicated are the isolation methods, isolation media, growth on NWC crude oil as the sole carbon source and ability to degrade hydrocarbons as determined by GC analysis (Fig. 2.3).

Species	Site	Isolation method[a]	Isolation Media[b]	Growth on crude oil[c]	Degradation of crude oil[d]
Acremonium sp.	BL, DV, NW	S, D	1, 2, 5	-	N
A. butyri	DV, NW, WG	S, D	1	-	N
A. kiliense	All	S, D	1, 4	-	N
A. strictum	DV	D	1	-	N
Alternaria alternata	BL, KS	S	1	-	N
Aspergillus sp. 1	KS	S, D	1, 2, 3, 4	+	G
Aspergillus sp. 2	WG	D	3	+	S
Aspergillus sp. 3	KS	S, K	1, 2, 4	-	N
A. fumigatus	WG	S, D	1, 4	+	G
A. niger	BL, DV, NW, WG	S, D	1, 2, 4, 5	-	N
A. cf. *niveus*	KS	D	1, 2, 5	+	G
A. cf. *tamarii*	KS	S	4	+	G
Aureobasidium pullulans	BL	S	1, 3	-	N
Beauveria bassiana	DV	D	2	+	P
Chaetomium globosum	BL, DV, WG	S, D	1, 4	-	N
Chalara sp.	BL	D	1	-	N
Cladosporium cladosporioides	DV, WG	S, D	3, 4	-	N
Exophiala jeanselmei	DV	S	4	-	N
Fusarium sp. 1	DV	S	1, 2, 3	+	P
Fusarium sp. 2	BL, DV	S, D	1, 3	+	P
F. avenaceum	DV, WG	S	1, 2	+	P-G
F. subglutinans	BL, DV, WG	S, D	1, 3	+	P-G
Geomyces pannorus	BL, DV, WG	S, D	4	-	N
Gliocladium cf. *catenulatum*	All	S, D	All	-	N
Gliomastix sp.	BL, WG	S, D	1, 3	-	N
Neosartorya fischeri	NW, WG	S, D	1, 3	+	G
Oidiodendron griseum	BL	S, D	1, 3	+	P-S
Paecilomyces marquandii	DV	S	4	-	N
P. variotii	DV, WG	S, D	2, 3	-	N
Paecilomyces sp.	BL	S	1, 5	+	G
Penicillium cf. *aurantiogriseum*	KS	S	1	+	P
P. cf. *decumbens*	DV, WG	S, D	1, 2, 3	+	G-S
P. cf. *glabrum*	DV, NW	D	1, 2	+	G
P. cf. *isariiforme*	DV	S, D	1, 4	+	G-S
P. cf. *janthinellum*	BL, DV, NW, WG	S, D	All	+	P-G

Continued

Table 2.1 continued

Species	Site	Isolation method[a]	Isolation Media[b]	Growth on crude oil[c]	Degradation of crude oil[d]
P. cf. *miczynaskii*	BL	D	1	+	P
P. cf. *minioluteum*	KS	S	6	+	G
P. pinophilum	KS	S	4, 5	+	G
P. cf. *purpurogenum*	DV, NW, WG	S, D	1, 2, 5	+	G
P. spinulosum	DV	D	1	+	G
P. thomii	BL, DV, WG	S, D	1, 2, 3, 4	+	G
P. cf. *variabile*	KS	S, D	1, 2, 4, 5	+	N
P. cf. *waksmanii*	BL, DV	D	4	+	G
Penicillium sp. 1	BL, DV, WG	S, D	1, 2, 4	+	G
Penicillium sp. 2	BL. NW	S, D	1, 2	+	G
Penicillium sp. 3	DV, WG	S, D	1, 2, 4	+	G
Penicillium sp. 4	BL, DV, WG	S, D	1, 4	+	G-S
Penicillium sp. 5	DV	S, D	1, 3	+	G
Penicillium sp. 6	DV	S, D	1, 3	+	G
Penicillium sp. 7	DV, NW	S, D	1, 4, 5	+	G
Penicillium sp. 8	KS	S	1, 5	+	P
Penicillium sp. 9	KS	S, D	1, 2, 5	+	G
Penicillium sp. 10	BL, DV, WG	S, D	1, 3, 4	+	G
Phialophora sp.	BL	S	2	+	P
Phialophora americana	NW	S, D	1, 2, 5	+	G, P
Phialophora verrucosa	NW	S	2	-	N
Phoma sp. 1	BL, WG, KS	S, D	1, 3, 5	-	N
Phoma sp. 2	BL	S	1, 2	-	N
Pseudallescheria boydii	BL, DV, NW, WG	S, D	All	+	G-S
Rhinocladiella atrovirens	KS	S	2	-	N
Rhizopus stolonifer	BL, KS	S	1, 2, 3	+	N
Sporothrix schenckii	DV	S	1	-	N
Trichoderma spp.	All	S, D	All	Not Tested	Not Tested
Ulocladium consortiale	BL	S	3	+	N

[a] Isolation methods: S, direct (sprinkle), D; dilution.

[b] The numbers 1 through 5 correspond to isolation media MEA+T, MEA+T+B, CMA+T, MYB and OAS, respectively.

[c] Growth of species incubated on NWC oil compared to growth without hydrocarbon; +, significant growth; -, insignificant growth.

[d] Degradation was based on GC profiles of residual NWC oil after 14 days incubation and was scored as significant (S; degradation of *n*-alkanes and isoprenoids), good (G, degradation of *n*-alkanes with little alteration of isoprenoids), partial (P, slight alteration of *n*-alkanes), or none (N, no difference from sterile control). See Fig. 2.3 for comparison.

Table 2.2. Species isolated from from five flare pit sites across Western Canada using dilution and direct isolation plating methods.

Norman Wells	Boundary Lake	Drayton Valley	Willesden Green	Kindersley
Acremonium sp.	*Acremonium* sp.	*Acremonium* sp.	*Acremonium butyri*	*Acremonium kiliense*
Acremonium butyri	*Acremonium kiliense*	*Acremonium butyri*	*Acremonium kiliense*	*Alternaria alternata*
Acremonium kiliense	*Alternaria alternata*	*Acremonium kiliense*	*Aspergillus fumigatus*	*Aspergillus* cf. *niveus*
Aspergillus niger	*Aspergillus niger*	*Acremonium strictum*	*Aspergillus niger*	*Aspergillus* cf. *tamarii*
Gliocladium cf. *catenulatum*	*Aureobasidium pullulans*	*Aspergillus niger*	*Aspergillus* sp. 2	*Aspergillus* sp. 1
Neosartorya fischeri	*Chaetomium globosum*	*Beauveria bassiana*	*Chaetomium globosum*	*Aspergillus* sp. 3
Penicillium cf. *glabrum*	*Chalara* sp.	*Chaetomium globosum*	*Cladosporium cladosporioides*	*Gliocladium* cf. *catenulatum*
Penicillium cf. *janthinellum*	*Fusarium subglutinans*	*Cladosporium cladosporoides*	*Fusarium avenaceum*	*Penicillium* cf. *aurantiogriseum*
Penicillium cf. *purpurogenum*	*Fusarium* sp. 2	*Exophiala jeanselmei*	*Fusarium subglutinans*	*Penicillium* cf. *minioluteum*
Penicillium sp. 1	*Geomyces pannorus*	*Fusarium avenaceum*	*Geomyces pannorus*	*Penicillium pinophilum*
Phialophora americana	*Gliocladium* cf. *catenulatum*	*Fusarium subglutinans*	*Gliocladium* cf. *catenulatum*	*Penicillium* cf. *variabile*
Phialophora verrucosa	*Gliomastix* sp.	*Fusarium* sp. 1	*Gliomastix* sp.	*Penicillium* sp. 8
Pseudallescheria boydii	*Oidiodendron griseum*	*Fusarium* sp. 2	*Neosartorya fischeri*	*Penicillium* sp. 9
Trichoderma spp.	*Penicillium* cf. *janthinellum*	*Geomyces pannorus*	*Paecilomyces variotii*	*Phoma* sp. 1
	Penicillium cf. *miczynaskii*	*Gliocladium* cf. *catenulatum*	*Penicillium* cf. *decumbens*	*Rhinocladiella atrovirens*
	Penicillium thomii	*Paecilomyces marquandii*	*Penicillium* cf. *janthinellum*	*Rhizopus stolonifer*
	Penicillium cf. *waksmanii*	*Paecilomyces variotii*	*Penicillium* cf. *purpurogenum*	*Trichoderma* spp.
	Penicillium sp. 1	*Penicillium* cf. *decumbens*	*Penicillium thomii*	
	Penicillium sp. 2	*Penicillium* cf. *glabrum*	*Penicillium* sp. 1	
	Penicillium sp. 4	*Penicillium* cf. *isariiforme*	*Penicillium* sp. 3	
	Penicillium sp. 10	*Penicillium* cf. *janthinellum*	*Penicillium* sp. 4	
	Penicillium sp. 11	*Penicillium* cf. *purpurogenum*	*Penicillium* sp. 10	
	Phialophora sp.	*Penicillium spinulosum*	*Phoma* sp. 1	
	Phoma sp. 1	*Penicillium thomii*	*Pseudallescheria boydii*	
	Phoma sp. 2	*Penicillium* cf. *waksmanii*	*Trichoderma* spp.	
	Pseudallescheria boydii	*Penicillium* sp. 1		
	Rhizopus stolonifer	*Penicillium* sp. 3		
	Trichoderma spp.	*Penicillium* sp. 4		
	Ulocladium consortiale	*Penicillium* sp. 5		
		Penicillium sp. 6		
		Penicillium sp. 7		
		Penicillium sp. 10		
		Pseudallescheria boydii		
		Sporothrix schenckii		
		Trichoderma spp.		

Table 2.3. Mean values of percent ^{14}C recovered in CO_2, aqueous, pentane, CH_2Cl_2 and biomass fractions after two weeks incubation of NWC oil (spiked with *n*-[1-^{14}C]-hexadecane) with filamentous fungi (see Fig. 2.2). All cultures were inoculated in triplicate and are presented in decreasing order of $^{14}CO_2$ production.

Culture and isolation number	% ^{14}C (± SD) recovered as $^{14}CO_2$ [a]	% ^{14}C (range) in aqueous [b] fraction	% ^{14}C (range) recovered in biomass [b]	% ^{14}C (range) recovered in CH_2Cl_2 [c]	% ^{14}C (range) in pentane [b] fraction	Total % ^{14}C (range) recovered
Aspergillus fumigatus S-WG1-4-1	**43.4** (± 5.3)	-	-	-	-	-
Aspergillus fumigatus D-WG1-4-1	**38.7** (± 9.5)	-	-	-	-	-
Phialophora americana S-NW1-6-1	**37.1** (± 19.5)	**7.2** (4.4-9.9)	**1.4** (0.9-2.0)	-	**29.8** (17.8-41.8)	**75.5** (70.4-80.6)
Penicillium thomii D-WG1-1-1	**37.0** (± 5.1)	-	-	-	-	-
Aspergillus fumigatus D-WG1-4-1	**34.4** (± 11.7)	**16.0** (10.8-24.3)	**11.6** (9.2-14.4)	**1.8** (1.0-2.6)	**22.3** (10.3-36.3)	**92.0** (86.3-97.7)
Oidiodendron griseum S-BL2-2-2	**31.7** (± 14.6)	**17.1** (10.6-23.6)	**3.1** (4.9-4.2)	**1.5** (0.8-2.1)	**34.0** (12.6-55.4)	**92.3** (89.7-94.9)
Oidiodendron griseum S-BL2-3-1	**29.0** (± 14.4)	**14.0** (12.5-15.5)	**3.7** (3.2-4.2)	**1.4** (1.0-1.8)	**35.0** (21.2-50.7)	**90.8** (85.9-95.7)
Pseudallescheria boydii D-NW2-7-2	**28.7** (± 3.5)	**8.5** (7.5-9.5)	**5.7** (5.2-6.1)	**3.9** (1.5-6.4)	**31.1** (29.9-32.4)	**78.0** (72.2-80.7)
Aspergillus fumigatus S-WG1-4-1	**21.2** (± 5.5)	**6.0** (3.3-8.6)	**9.0** (6.8-10.8)	**9.3** (9.0-9.6)	**45.3** (26.6-45.3)	**93.0** (76.5-109.6)
Neosartorya fischeri S-NW1-1-2	**13.6** (± 7.6)	-	-	-	-	-
Neosartorya fischeri S-NW1-1-2	**5.2** (± 1.3)	**3.6** (3.4-3.7)	**11.0** (9.7-13.1)	**9.6** (9.0-10.2)	**24.0** (18.7-29.3)	**51.6** (47.5-55.7)
Oidiodendron griseum S-BL2-4-2	**5.1** (± 4.1)	**3.0** (2.9-3.1)	**6.9** (2.4-9.2)	**2.0** (1.8-2.2)	**86.1** (78.4-93.7)	**103.1** (88.8-110.4)
Penicillium thomii D-WG1-1-1	**3.7** (± 0.6)	**4.8** (2.6-7.0)	**4.0** (3.8-4.1)	**4.6** (4.2-5.0)	**66.7** (65.7-67.6)	**83.6** (81.2-86.0)
Fusarium subglutinans D-DV1-2-1	**3.3** (± 3.8)	**2.8** (1.8-3.8)	**1.2** (0.7-1.6)	**4.4** (4.0-4.8)	**76.9** (73.7-80.2)	**89.5** (88.2-90.8)
Aspergillus niger S-NW1-7-1	**1.9** (± 2.0)	-	-	-	-	-
Fusarium sp. 2 D-BL1-3-2	**1.3** (± 0.7)	**3.9** (1.8-5.9)	**3.3** (2.6-3.9)	**5.3** (4.6-6.1)	**76.7** (59.8-93.6)	**90.8** (72.1-109.6)
Fusarium avenaceum S-WG1-2-1	**1.2** (± 0.4)	**4.6** (3.1-6.1)	**3.5** (2.4-4.6)	**4.6** (4.1-5.2)	**69.6** (69.1-70.1)	**83.5** (80.3-86.8)

(Continued)

Table 2.3 continued

Culture and isolation number	% ^{14}C (± SD) recovered as $^{14}CO_2$ [a]	% ^{14}C (range) in aqueous [b] fraction	% ^{14}C (range) recovered in biomass [b]	% ^{14}C (range) recovered in CH_2Cl_2 [c]	% ^{14}C (range) in pentane [b] fraction	Total % ^{14}C (range) recovered
****Paecilomyces variotii* D-DV1-2-2	**1.1** (± 0.8)	-	-	-	-	-
****Geomyces pannorus* D-BL1-6-1	**1.0** (± 0.5)	-	-	-	-	-
****Phialophora* sp. D-NW2-1-2	**1.0** (± 0.3)	-	-	-	-	-
****Chaetomium globosum* D-DV2-1-1	**0.8** (± 0.7)	-	-	-	-	-
Fusarium sp. 1 S-WG1-4-1	**0.6** (± 0.3)	**1.9** (1.3-2.4)	**7.6** (7.3-7.8)	**6.9** (5.6-8.2)	**62.7** (57.6-67.8)	**80.0** (73.0-87.0)
****Ulocladium consortiale* D-BL1-3-1	**0.6** (± 0.2)	-	-	-	-	-
****Rhizopus stolonifer* S-BL2-1-2	**0.5** (± 0.2)	-	-	-	-	-
Sterile control [d]	**0.1** (± 0.0)	**2.0** (± 0.7)	-	-	**83.0** (± 16.0)	**85.1** (± 15.5)

[a] Values are based on recovery of $^{14}CO_2$ from all replicates.

[b] Values are based on recovery of ^{14}C from two randomly selected replicates.

[c] The percent ^{14}C recovered with CH_2Cl_2 was extracted from the mycelial mass after removal from the aqueous and pentane fractions (Fig. 2.2).

[d] Mean value and standard deviation based on four samples.

* Measured only $^{14}CO_2$; cultures were inoculated using mycelium or conidia (not as an agar plug).

-, not determined

Table 2.4. Mean values of percent ^{14}C recovered in CO_2, aqueous, pentane, CH_2Cl_2 and biomass fractions after two weeks incubation of NWC oil (spiked with [9-^{14}C]-phenanthrene) with filamentous fungi (see Fig. 2.2). All cultures were incubated in triplicate and are presented in decreasing order of percent ^{14}C recovered in the aqueous fraction.

Culture and isolation number	% ^{14}C (range) in aqueous[a] fraction	% ^{14}C (± SD) recovered as $^{14}CO_2$[b]	% ^{14}C (range) recovered in biomass[a]	% ^{14}C (range) recovered in CH_2Cl_2[c]	% ^{14}C (range) in pentane[a] fraction	Total % ^{14}C (range) recovered
Fusarium avenaceum	**8.6**	**0.1**	**2.9**	**4.8**	**99.1**	**122.1**
S-WG1-2-1	(6.2-11.2)	(± 0.0)	(2.8-3.0)	(4.6-4.9)	(88.1-106.8)	(118.2-126.0)
Pseudallescheria boydii	**8.3**	**0.3**	**12.5**	**6.6**	**71.3**	**96.9**
D-NW2-7-2	(7.4-9.2)	(± 0.1)	(10.3-14.6)	(4.1-9.1)	(54.9-87.6)	(86.3-107.5)
Penicillium thomii	**7.0**	**0.1**	**8.4**	**5.4**	**91.9**	**110.7**
D-WG1-1-1	(6.2-7.8)	(± 0.0)	(2.7-12.5)	(4.9-5.8)	(85.7-98.1)	(109.3-112.1)
Neosartorya fischeri	**6.9**	**0.1**	**16.1**	**11.8**	**39.9**	**74.8**
S-NW1-1-2	(6.7-7.1)	(± 0.0)	(12.7-19.6)	(10.7-12.9)	(32.4-47.4)	(69.5-80.2)
Oidiodendron griseum	**6.4**	**0.3**	**2.6**	**8.2**	**62.2**	**79.7**
S-BL2-4-2	(6.4-6.4)	(± 0.1)	(2.5-2.7)	(3.7-12.6)	(21.2-103.3)	(43.4-116.0)
Aspergillus fumigatus	**5.3**	**0.1**	**6.9**	**7.8**	**40.1**	**60.3**
D-WG1-4-1C	(4.6-5.9)	(± 0.0)	(6.1-7.8)	(6.8-8.7)	(28.5-51.8)	(51.1-69.5)
Aspergillus fumigatus	**4.8**	**0.1**	**7.1**	**3.9**	**57.4**	**72.8**
D-WG1-4-1F	(4.6-4.9)	(± 0.0)	(6.7-8.0)	(2.9-4.8)	(55.3-59.6)	(71.6-73.9)
Sterile control[d]	**5.3**	**0.1**		-	**77.7**	**83.1**
	(± 1.4)	(± 0.0)			(± 13.6)	(± 14.3)

[a] Values are based on recovery of ^{14}C from two randomly selected replicates.
[b] Values are based on recovery of $^{14}CO_2$ from all replicates.
[c] The percent ^{14}C recovered with CH_2Cl_2 was extracted from the mycelial mass after removal from the aqueous and pentane fractions (Fig. 2.2).
[d] Mean value and standard deviation based on four samples.

Table 2.5. Systematic arrangement of some hyphomycete genera according to their known affinity with teleomorphic genera within the phylum Ascomycota.

Order	Family	Genus	Anamorph Genus
Dothidiales	Leptosphaeriaceae	*Ophiobolus*	*Phialophora*
Familia Incertae Sedis	Magnaporthaceae	*Gaeumannomyces*	*Phialophora*
Eurotiales	Trichocomaceae	*Byssochlamys*	*Paecilomyces*
		Eupenicillium	*Penicillium*
		Talaromyces	*Penicillium*
		Emericella	*Aspergillus*
		Eurotium	*Aspergillus*
		Neosartorya	*Aspergillus*
Hypocreales	Hypocreaceae	*Gibberella*	*Fusarium*
		Nectria	*Fusarium*
Microascales	Microascaceae	*Pseudallescheria*	*Graphium*
		Pseudallescheria	*Scedosporium*
Onygenales	Myxotrichaceae	*Byssoascus*	*Oidiodendron*
		Myxotrichum	*Oidiodendron*

CHAPTER 3

Degradation of hydrocarbons in crude oil by the ascomycete *Pseudallescheria boydii* (Microascaceae)[1]

INTRODUCTION

Recently, our attention was directed to several strains of mould that appeared to thrive in soils heavily contaminated with petroleum. In pure culture, a *Graphium* and *Scedosporium* state were produced that were morphologically similar to the anamorphs *of Pseudallescheria boydii* (Negroni & Fischer) McGinnis, Padhye & Ajello. Subsequently, after a long incubation period, two strains formed cleistothecia typical of *Pseudallescheria boydii* (McGinnis et al. 1982). *Pseudallescheria boydii* has a worldwide distribution and is recovered from nutrient-rich poorly aerated soils, polluted water, decayed wood, manure, and sewage sludge (Ajello 1952, 1980; Bell 1976; Cooke and Kabler 1955; de Hoog et al. 1994). It is also a well-known opportunistic pathogen of humans and other animals (Rippon 1988).

When isolated in pure culture, *Pseudallescheria boydii* can be difficult to identify because cleistothecia form only after prolonged incubation or not at all (Gordon 1956; Bell 1976) and isolates may lose the ability to form cleistothecia with characteristic anamorphic (asexual conidial) states that are assignable to the form genera *Graphium* and *Scedosporium* (Campbell and Smith 1982; Hironaga and Watanabe 1980). Neither of

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these genera are specific to *Pseudallescheria* and, consequently, reports of *Graphium* species and *Scedosporium* species in the literature are difficult to interpret. For example, *Graphium* species have been reported from soil (Barren 1968), woody materials (Morris 1963), and raw sewage (Davies et al. 1973; Zajic et al. 1969), but it is uncertain if these isolates represent anamorphs of *Pseudallescheria boydii* or other teleomorph genera such as species of *Ophiostoma, Petriella, Kernia,* and *Chaetosphaeria* (Seifert and Okada 1993; Carmichael et al. 1980).

Our isolates were obtained from oil-well flare pits, which are areas where hydrocarbon wastes from gas and oil facilities are burned. The soils in these flare pits are saturated with pyrolized petroleum residues. The profuse growth of *Pseudallescheria boydii* on soil samples from these sites suggests that the medium to heavy molecular weight petroleum compounds are utilized as a source of carbon. An unidentified isolate of *Graphium* was reported as having the ability to degrade low molecular weight alkanes, such as ethane, propane, and *n*-butane (Zajic et al. 1969; Davies et al. 1973), but was unable to utilize higher molecular weight hydrocarbons, such as those found in crude oil or pyrolized petroleum residues (Volesky and Zajic 1970). *Graphium rubrum* Rumbold and *Graphium fructicolum* Marchal & E. J. Marchal were observed to utilize higher molecular weight *n*-alkanes (Lowery et al. 1968; Llanos and Kjoller 1976). *Graphium fructicolum* was repeatedly isolated from treated oil-contaminated soils and was almost entirely absent from control soils (Llanos and Kjoller 1976), indicating that the presence of hydrocarbons favoured its growth.

The taxonomic relatedness of six strains with one or more spore states resembling *Pseudallescheria boydii* was assessed and, using gas chromatography, their relative abilities to degrade a range of crude oil hydrocarbons were determined.

MATERIALS AND METHODS

Hydrocarbon-contaminated soil samples were collected from flare pits at Boundary Lake, BC, Drayton Valley, AB, and Willesden Green, AB. Approximately 10 g of soil was sprinkled on corn meal agar (CMA; Difco), malt extract agar (MEA; Difco), and potato dextrose agar (PDA; Difco). All media contained 100 mg oxytetracycline/L and 2 mg benomyl/L to control the growth of bacteria and minimize the development of various hyphomycetes (moulds). *Pseudallescheria boydii* was established from the primary isolation plates by transferring conidia or mycelium to MEA with oxytetracycline. On the primary isolation plates, colonies of *Pseudallescheria boydii* were distinguished from other fungi present by white to gray floccose aerial mycelia and (or) the presence of synnemata.

The isolates from flare pit soils were compared using morphological, molecular, and biochemical data with a strain expressing similar *Graphium* and *Scedosporium* states obtained from the American Type Culture Collection (ATCC) (Zajic et al. 1969) and with ascocarpic strains of *Pseudallescheria boydii* (DAOM 148868; Bell 1976) and *Petriella sordida* (Zukal) G. L. Barren and J. C. Gilman (DAOM 162159) obtained from the Canadian Collection of Fungus Cultures (CCFC). All strains examined (Table 3.1) are deposited in the University of Alberta Microfungus Collection and Herbarium (UAMH).

The six *Pseudallescheria boydii* strains (Table 3.1) were inoculated onto PDA, PDA plus 2 mg benomyl/L (PDA+B), and Mycosel agar (MYC, Becton Dickinson) plates and incubated at 25 °C and on PDA plates incubated at 37 °C. Colonial appearance, colony diameter, and ability to grow at 37 °C were observed following a 14-day incubation period. Colony diameters were measured in cultures prepared by inserting a sterile straight needle into a suspension of conidia and (or) ascospores prepared for each strain in semisolid detergent agar (Pitt 1973) and then stab inoculating them into the center of Petri plates containing 35 mL of PDA, PDA+B, or MYC (Abbott et al. 1995). Colony features were recorded and colours were determined using the colour standards of Kornerup and Wanscher (1978). Morphology of conidial structures was examined by slide culture preparations (Sigler 1992). Mounts of ascocarps and synnemata were prepared in polyvinyl alcohol or lachtofuchsin mounting medium (Sigler 1992) and ascospores were observed in squash mount preparations. To induce ascocarp formation, cultures were incubated on MEA and oatmeal agar (OAT; Weitzman and Silva-Hutner 1967) and incubated with ultraviolet light (both black light and natural daylight) (Seifert et al. 1993). Selected specimens were prepared by established methods of fixation in glutaraldehyde, post-fixation in osmium tetroxide, drying to the critical point, and examination with a Cambridge Stereoscan S-250 scanning electron microscope (SEM).

For restriction fragment length polymorphism (RFLP) analysis, the internally transcribed spacer region (ITS) of the nuclear ribosomal DNA (rDNA) of *Pseudallescheria boydii* strains UAMH 8598, UAMH 8792, UAMH 8794, and UAMH 8897 and *Petriella sordida* strain UAMH 8695 (for comparison) was amplified using the PCR primers ITS 1F and ITS 4 (Gardes and Bruns 1993; White et al. 1990) and digested

with the restriction enzymes *Alu*I, *Hha*I, *Hinf*I, and *Rsa*I. Extraction, amplification, and restriction followed procedures described by Gardes and Bruns (1996) and Kernaghan et al. (1997); data were analyzed by Gel Pro software (Media Cybernetics, Silver Springs, Md.).

For gas chromatographic analysis of residual hydrocarbons, each strain was inoculated onto 20 mL of mineral salts agar (4.4 g K_2HPO_4, 1.7 g KH_2PO_4, 2.1 g NH_4Cl, and 15 g Noble agar (Difco) per litre of double-distilled water and 10 mL of salt solution [19.5 g $MgSO_4 \cdot 7H_2O$, 5.0 g $MnSO_4 \cdot H_2O$, 5.0 g $FeSO_4,7H_2O$, 0.3 g $CaCl_2 \cdot 2H_20$, and 1.0 g ascorbic acid per litre of double-distilled water]) in 125-mL flasks and incubated at room temperature (approximately 22 °C) for 1 week to establish mycelial growth. For each strain, three replicates were prepared for each extraction period (7, 14, and 21 days) along with two sterile control replicates. For Day 0, there was one replicate for each strain and a sterile control. Day 0 was defined as the time when 0.05 mL of sterile nutrient solution (25.0 g K_2HPO_4, 50.0 g NH_4Cl, and 1 00.0 g KNO_3 per litre of double-distilled water) and 0.10 mL of Prudhoe Bay Crude oil as sole carbon source were added to each 125-mL flask. Cultures were incubated for 21 days at room temperature in the dark to prevent photooxidation.

At 1-week intervals, beginning at Day 0, 0.025 mL of stock surrogate standard solution (containing chrysene and squalane) was added to three randomly selected flasks and residual oil was recovered from replicate cultures using 2 mL of CH_2Cl_2 (spectrophotometric grade). Water in the extract from condensation on the agar was removed by adding anhydrous sodium sulfate and the extracts were concentrated under N_2 to 0.5 mL. Day 21 extracts were fractionated by liquid column chromatography on

activated silica gel (Fedorak and Westlake 1981) into saturate and aromatic fractions and analyzed by gas chromatography (GC; Hewlett Packard Model 5890 with DB-5 fused silica column [J+W Scientific], 25 m x 0.2 mm). The GC followed a temperature program of 90 °C for 4 min, and then 4 °C/min to 250 °C, which was held for 16 min (Foght et al. 1990).

Following results of GC analysis from the crude oil incubation, UAMH 8792 was chosen as a representative strain of the flare pit isolates to compare to UAMH 8598. The two strains were inoculated on mineral salts agar and incubated at room temperature (approximately 22 °C) in the dark with gasoline vapors for 7 days to determine if relatively low molecular-weight hydrocarbons, including volatile *n*-alkanes, could be utilized as a carbon source. Parallel controls of both strains incubated without gasoline were included for comparison.

RESULTS

ISOLATION AND CHARACTERISTICS OF STRAINS

Colonies of *Pseudallescheria boydii* were observed on all primary isolation media containing flare pit soils from Drayton Valley, Willesden Green, and Boundary Lake. After 14 days, colonies (obverse) on PDA at 25 °C were grey brown (4B 1-4B2; Kornerup and Wanscher 1978) to pallid grey (1B1-3B1-3B2), especially at the margin, often slightly darker (4B3-4C3-4D3) on MYC and PDA at 37 °C, floccose to woolly fasciculate, shallowly convex, and with the margin entire or slightly fimbriate (Fig. 3.11). Colonies of UAMH 8598 were pale and extremely floccose, while cultures producing cleistothecia were darker and less floccose and had more uniform mycelial growth across the medium. Ultraviolet light enhanced cleistothecium formation. A clear to brown exudate was frequently observed, especially at 37 °C. The *Pseudallescheria boydii* teleomorph and *Graphium* state were not produced at the same time in culture.

Cleistothecia were scattered throughout the colony in the aerial mycelium and submerged in the agar. They were black, globose, 60-140 µm in diameter, lacked appendages, and had a peridium of *textura epidermoidea* with cells 2-6 µm in diameter (Fig. 3.2). Asci were evanescent and rarely seen, subglobose, 14-16 x 1014 µm in diameter, and contained eight reddish brown, smooth-walled, ellipsoidal ascospores (7.5-8.5 x 4.5-5.5 µm) with de Bary bubbles in a large proportion of spores in the polyvinyl alcohol and lactofuchsin mounting media (Fig. 3.2, inset). The *Scedosporium* state consisted of lightly pigmented conidia (6-13 x 3-6 µm), which varied from

subglobose, ellipsoidal to clavate and had a truncate base (Fig. 3.3) and a distinct attachment scar. They were produced singly or annellidically on hyaline, lateral, or terminal conidiogenous hyphae of uniform diameter and variable length (5-50 x 3-4 μm). The *Graphium* state was recognized by dematiaceous synnemata, 160-225 μm tall, that produced slimy masses of cylindrical (6-8 x 2-3 μm) hyaline conidia (Fig. 3.4). SEM (Fig. 3.5) revealed distinct annellidic conidiogenous cells (15-20 x 2.5-3 μm) on the conidiophores with attachment scars similar to those of the *Scedosporium* state.

As shown in Table 3.2, growth of the strains at 25 and 37°C varied and formation of teleomorphic and anamorphic states was inconsistent among the six strains. The addition of benomyl to the medium did not affect colony size (±3 mm), and all strains were resistant to cycloheximide as demonstrated by growth on MYC, although growth of one strain (UAMH 8598) was markedly reduced (Table 3.2).

RFLP ANALYSIS

Identical RFLP profiles were observed among the four strains of *Pseudallescheria boydii* for each restriction enzyme: *Alu*I (112 and 417 bp), *Hha*I (105, 132, and 205 bp), *Hinf*I (130, 196, and 326 bp), and *Rsa*I (651 bp). This indicated that restriction sites were the same for all strains within the amplified ITS region. *Petriella sordida,* a closely related taxon used to assess the sensitivity of the RFLP analysis, displayed a different profile: *Alu*1 (134, 168, and 301 bp). *Hha*I (104, 136, and 329 bp), *Hinf*I (doublet at 315 bp), and *Rsa*I (626 bp). Fragment sizes (bp) are accurate to ±3%.

GC ANALYSIS

By Day 7, colonies of UAMH 8792, 8793, and 8794 on mineral agar plus oil exhibited considerable growth, forming a leathery layer of oil and mycelium. As a consequence, the oily residue did not readily separate from the mycelia during solvent extraction with CH_2Cl_2. In contrast, UAMH 8598 demonstrated little growth and the oily residue was easily extracted. Residual oil from both UAMH 8598 and sterile controls appeared similar in viscosity to that at Day 0.

The saturate profile of fractionated residual oil showed that *n*-alkanes had been removed from oil incubated with isolates from the hydrocarbon-contaminated soil (UAMH 8792, 8793, and 8794), whereas UAMH 8598 (isolated from raw sewage) showed no *n*-alkane degradation. UAMH 8792 showed complete removal of *n*-C_{12} to *n*-C_{21} and partial removal of *n*-C_{22} to *n*-C_{26} in comparison with the sterile control GC profile (Fig. 3.6). There was a slight reduction in peak height of the branched-chain alkane isoprenoids (pristane and phytane) indicating partial degradation, with the peak ratios of pristane and phytane to the surrogate standard squalane lower than the control (Table 3.3). A similar saturate profile was observed with UAMH 8794 (not shown), with identical peak removal and peak reduction indicating degradation of those compounds. UAMH 8793 showed the greatest *n*-alkane removal of the four strains incubated on the crude oil (Fig. 3.6), with the saturates *n*-C_{12} to *n*-C_{25} completely degraded and *n*-C_{26} to *n*-C_{28} considerably reduced. Pristane and phytane were degraded the most in comparison with the other strains, having the lowest pristane and phytane to squalane ratios (Table 3.3).

GC analysis did not indicate any degradation of the aromatic fraction (Fig 3.7). Profiles of all four strains (represented by UAMH 8794) incubated on Prudhoe Bay Crude oil were almost identical to the control. However, both UAMH 8792 and 8794 did exhibit a peak not present in the profile of the other two strains or the control. The additional peak, found between those of the C_2 and C_3 naphthalenes (Fig. 3.7), was not identified.

UAMH 8598 inoculated on mineral agar with a gasoline vapor carbon source grew significantly better than the parallel control incubated without gasoline. Floccose white aerial mycelia developed in the presence of gasoline compared with scant surface mycelia in the control. UAMH 8792 did not grow appreciably in the presence of gasoline vapors and resembled the control.

DISCUSSION

Considerable variation in morphology and physiology was present among the six strains of *Pseudallescheria boydii* (Table 3.2), but the production of at least two of the three characteristic sporogenous states provided sufficient evidence of conspecificity, an observation supported by molecular analysis. All strains consistently produced the *Scedosporium* state. Cleistothecia were produced inconsistently in UAMH 8792, with only some subcultures developing ascocarps. Further subculture from cleistothecium-producing sectors of the colony enhanced ascocarp development. Although ascocarpic strains produced cleistothecia on all media under varied conditions, ascocarp production was greatest on minimal media, especially OAT, and with growth under ultraviolet light. Synnemata production was sporadic, most abundant on PDA and MEA but not observed

in the same culture plate as the ascocarps. Gordon (1956) suggests that strain variation is genetic, but it appears that environmental stimuli may also influence cleistothecial and synnematal production. The polymorphism of this fungus adds to the taxonomic confusion with different strains potentially producing one to three different states (de Hoog et al. 1994; Hironaga and Watanabe 1980). Resistance to benomyl is useful to differentiate *Pseudallescheria boydii* and related species from morphologically similar anamorphic states of *Ophiostoma* species (S. Abbott, unpublished data).

Growth at 37 °C was an inconsistent characteristic among the strains with two growing faster, two slower, and two unchanged in growth rate at the higher temperature (Table 3.2). However, the data demonstrate that all strains are capable of growth at 37 °C. Similarly, all strains grew on MYC, although growth of UAMH 8598 was reduced. De Hoog et al. (1994) observed comparable results with strains of *Pseudallescheria boydii* growing on a similar cycloheximide-containing medium: all strains grew on solid medium containing cycloheximide, but there was variable growth of strains in liquid culture containing cycloheximide. Considering the genetic variation in this species (Gordon 1956) and the tendency for each strain to exhibit different sporogenous states in response to changing environmental parameters, it is understandable that the specific name for this apparently common inhabitant of oil-soaked soils has not been reported previously.

Utilization of a wide range of linear saturated hydrocarbons by some strains of *Pseudallescheria boydii* potentially makes this ascomycete a good candidate in a consortium of microorganisms for bioremediation. UAMH 8598 (= ATCC 5840) previously known as *Graphium* sp. and here shown to be conspecific with

Pseudallescheria boydii, degraded volatile saturates such as ethane, propane, and *n*-butane (Zajic et al. 1969), but no growth was observed on hydrocarbons of higher molecular weight than *n*-butane. Further tests on the same strain showed that methane alone was not oxidized, but may be co-oxidized in the presence of ethane (Volesky and Zajic 1970). *n*-Alkynes and *n*-alkenes (acetylene, 1-butyne, ethylene, and propylene) inhibited the growth of *n*-alkane-utilizing *Graphium* (Curry et al. 1996). In addition, a recent study by Hardison et al. (1997) showed that this isolate can also degrade the gasoline oxygenate, methyl *tert*-butyl ether, and may be applied for the remediation of methyl *tert*-butyl ether contaminated soils.

In addition to the ability to utilize gaseous hydrocarbons present in gasoline, at least some strains of *Pseudallescheria boydii* (UAMH 8791, UAMH 8792, UAMH 8793; UAMH 8794) can degrade mid- to higher molecular weight alkanes *(n*-C_{12} to *n*-C_{26}*)* found in crude oil. This is of particular importance because most oil-contaminated sites requiring reclamation have been weathered and contain only the higher molecular weight saturates, including the *n*-alkanes degradable by these strains of *Pseudallescheria boydii.* While the change in the saturate GC profile after incubation with *Pseudallescheria boydii* can be interpreted as carbon mineralization, an alternative explanation would be that the organism is transforming the *n*-alkanes into intermediate compounds less toxic to growth. However, considering that the fungus grows luxuriantly when crude oil is the only carbon source, it is more likely the process is degradative with the release of CO_2, rather than the process of detoxification.

Other *Graphium* species (reported as G. *rubrum* and G. *fructicolum;* Lowery et al. 1968; Llanos and Kjoller 1976) also have been observed to utilize higher molecular

weight *n*-alkanes (*n*-C_{10} to *n*-C_{16}). *Graphium putredinis,* a species complex with a number of teleomorphic affinities including *Pseudallescheria* (Ellis 1971; Seifert and Okada 1993) was observed to degrade saturates and aromatics and, to a lesser extent, resins and asphaltenes (Oudot et al. 1993). As observed with the *Pseudallescheria boydii* strains isolated from flare pits, *G. putredinis* showed a high degree of variability in its ability to degrade hydrocarbons. While one strain was able to degrade a wide range of hydrocarbons, a second isolate exhibited much less activity (Oudot et al. 1993).

Pseudallescheria boydii has adapted to environments of low oxygen levels and high salt concentrations (2% NaCI and 5% $MgC1_2$) (de Hoog et al. 1994). Because these conditions are found in water-logged hydrocarbon-contaminated soils that often have low oxygen levels and high salt content owing to the brine used in drilling, *Pseudallescheria boydii* may be a candidate for use in bioremediation. The presence of hydrocarbons may naturally favor the development of *Pseudallescheria boydii,* as was seen with G. *fructicolum* in hydrocarbon-contaminated soils (Llanos and Kjoller 1976) and indicated by this study. Tolerance of the *Pseudallescheria boydii* strains isolated from flare pits to elevated temperatures may influence their potential use in various bioremediation strategies (e.g., ground temperature versus higher temperature oil-composting biopiles). Conversely, further biodogradative studies (reported in Chapter 2) at low temperatures more representative of surface soils.

Pseudallescheria boydii is a ubiquitous saprobic fungus commonly isolated from soil and waste material (Cooke and Kabler 1955; Ajello 1980), which accounts for the spectrum of opportunistic diseases (pseudallescheriosis) that it causes (Rippon 1988). Cases of infection owing to *Pseudallescheria boydii* have been reported in Alberta (e.g.,

Dowding 1935) and many other isolates from clinical and natural sources in Alberta are known (over 20 strains in UAMH have been examined). Bell (1978) demonstrated considerable variation in pathogenicity of strains, with an isolate from manure significantly less virulent than one isolated from a human mycetoma. The virulence of the strains isolated from flare pits remains to be tested.

The apparent widespread occurrence of *Pseudallescheria boydii* indicates that it may already be an inherent part of petroleum-contaminated soils in western Canada. However, the organism's behaviour under typical *in situ* conditions remains unclear, including its response to various nutrient conditions, low and fluctuating temperatures, and the presence of other microbial populations. *Pseudallescheria boydii,* like many fungal pathogens, is difficult to treat *in vivo* and is resistant to antifungals such as amphotericin. However, this species is susceptible to the azole antifungal antibiotics, with miconazole, ketoconazole, and intraconazole having been proven effective in some cases (Richardson and Warnock 1993; Rippon 1988). Because the (variable) pathogenicity of the natural flare pit strains has yet to be determined, we recommend caution when dealing with *Pseudallescheria boydii.*

However, under properly controlled conditions, selected non-pathogenic strains of *Pseudallescheria boydii* may have the potential to be used safely as an integral and effective part of the intrinsic bioremediation process.

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Table 3.1. Substrate, location, and isolation data for the *Pseudallescheria boydii* and *Petriellidium sordida* strains examined.

Strain	Source
Pseudallescheria boydii	
UAMH 8791	Hydrocarbon-contaminated flare pit soil; Willesden Green northeast of Rocky Mountain House. AB, C. Zelmer: 13 Apr. 1994.
UAMH 8792	Hydrocarbon-contaminated flare pit soil: Willesden Green northeast of Rocky Mountain House, AB, T. April: 16 Aug. 1995.
UAMH 8793	Hydrocarbon-contaminated flare pit soil; Cynthia west of Drayton Valley, AB; T. April; 16 Aug. 1995.
UAMH 8794	Hydrocarbon-contaminated flare pit soil; Boundary Lake, BC; T. April; 21 June 1996.
UAMH 8598	Raw sewage; London, ON; Zajic et al. (1969); 1967; obtained as ATCC 58400 (= NRRL 3915).
UAMH 8897	Fresh faeces of beef cattle; Lethbridge, AB; R. Bell; 1975; obtained from CCFC as DAOM 148868.
Petriellidium sordida	
UAMH 8695	Twigs of apple; Kentville, NS; C.O. Gourley; 1976; obtained from CCFC as DAOM 162159.

Table 3.2. Colony diameters, temperature tolerance, and sporulation in six strains of *Pseudallescheria boydii* after incubation at 25 or 37°C for 14 days on PDA, PDA+B, or MYC.

Strain	Colony diameter at 14 days (mm)				Ascomata	*Graphium* state	*Scedosporium* state
	PDA (25°C)	PDA+B (25°C)	MYC (25°C)	PDA (37°C)			
UAMH 8791	58	57	42	85	-	+	+
UAMH 8792	55	55	45	56	+	+	+
UAMH 8793	64	65	52	63	-	+	+
UAMH 8794	58	55	46	80	+	-	+
UAMH 8598	70	70	10	47	-	+	+
UAMH 8897	55	55	42	43	+	-	+

Table 3.3. Peak height ratios of the isoprenoids, pristane and phytane, to the surrogate standard squalane in residual oil extracted from cultures of UAMH 8792, UAMH 8793, UAMH 8794, UAMH 8598, and a sterile control.

Strain	Pristane to squalane ratio[a]	Phytane to squalane ratio
UAMH 8792	1.25±0.23	0.92±0.13
UAMH 8793	0.80±0.18	0.67±0.20
UAMH 8794	1.09±0.27	0.79±0.11
UAMH 8598	1.55±0.36	1.09±0.30
Sterile control	1.33±0.49	0.84±0.39

[a]Mean ± 1 SD; $n = 3$, except for the sterile control, $n = 2$.

Figures 3.1 - 3.5. *Pseudallescheria boydii* UAMH 8794. **Figure 3.1**. Colony on PDA incubated at 37°C for 14 days. Scale bar = 20 mm. **Figure 3.2**. Ruptured cleistothecium with ascospores and surrounding hyphae. Scale bar = 40 µm. The inset shows ascospores with de Bary bubbles. Scale bar = 10 µm. **Figure 3.3**. Scanning electron micrograph of conidia of the *Scedosporium* state. Scale bar = 2 µm. **Figure 3.4**. Synnematous conidiophore of Graphium state (arrowhead) with the synanamorphic Scedosporium state (double arrowheads). Scale bar = 25 µm. **Figure 3.5**. Scanning electron micrograph of conidia and annellations (arrowheads) of the *Graphium* state. Scale = 4 µm.

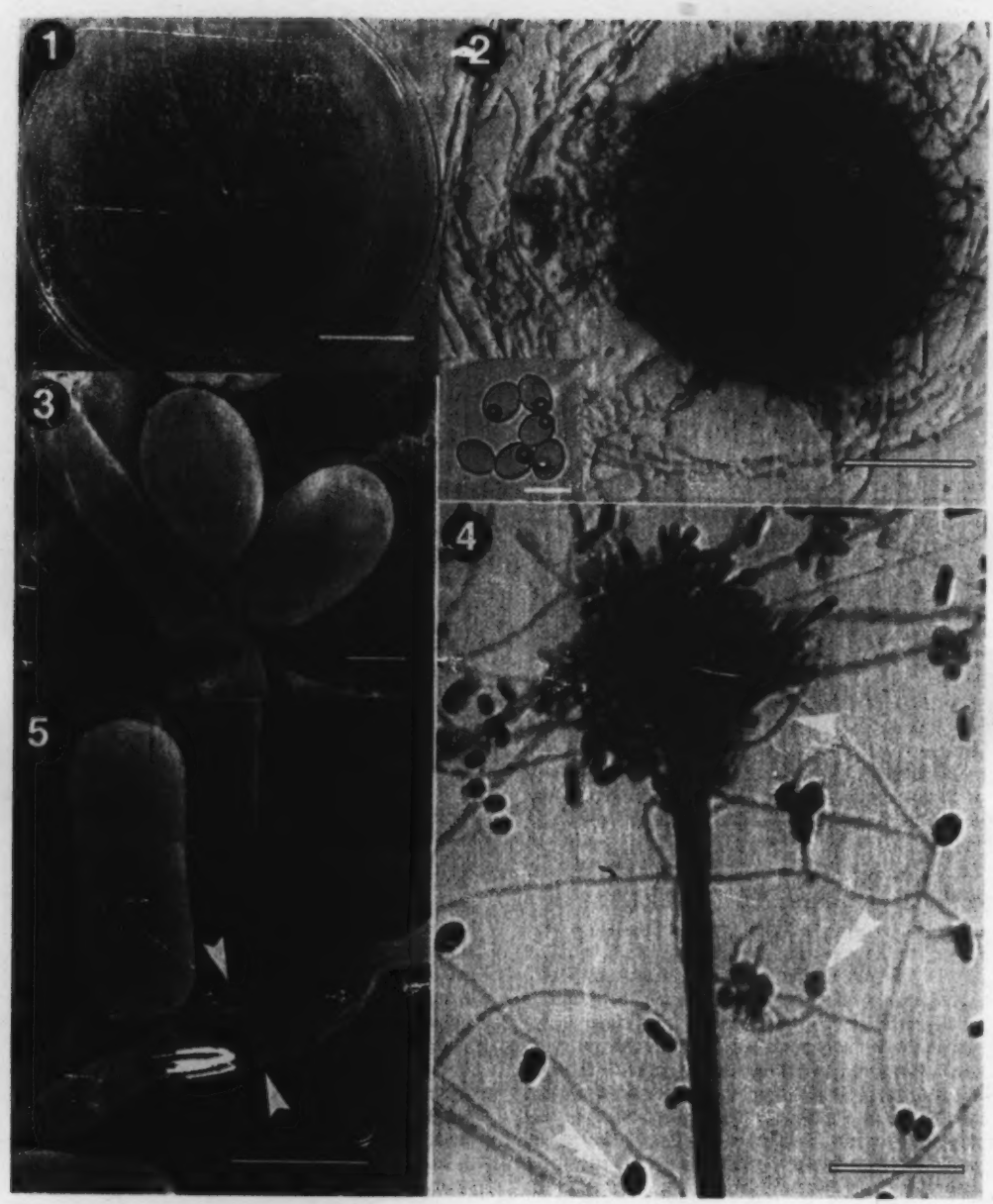

1
2
3
4
5

Figure 3.6. Gas chromatographic profiles of the saturate fraction of Prudhoe Bay Crude oil following a 3-week incubation with strains of *Pseudallescheria boydii*. **A** - Sterile control, no hydrocarbon degradation, similar to profile observed with UAMH 8598; **B** - UAMH 8792 showing hydrocarbon degradation, similar to profile observed with UAMH 8794; **C** - UAMH 8793, showing greatest degree of hydrocarbon degradation among *Pseudallescheria boydii* strains. S, squalane (surrogate standard); Pr, pristane; Ph, phytane; n-C_{16}, hexadecane: n-C_{24}, tetracosane.

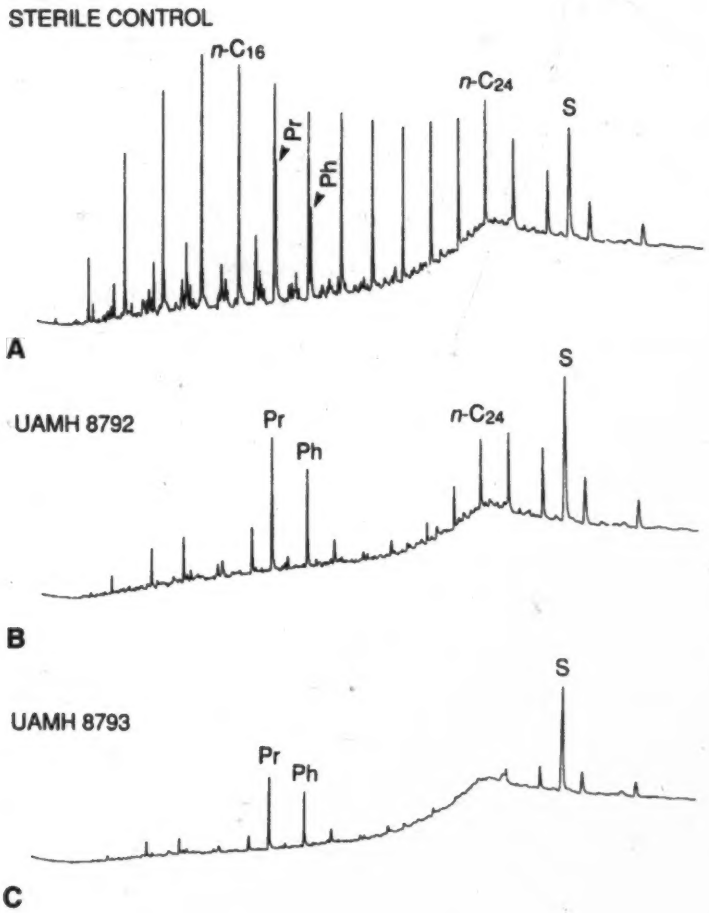
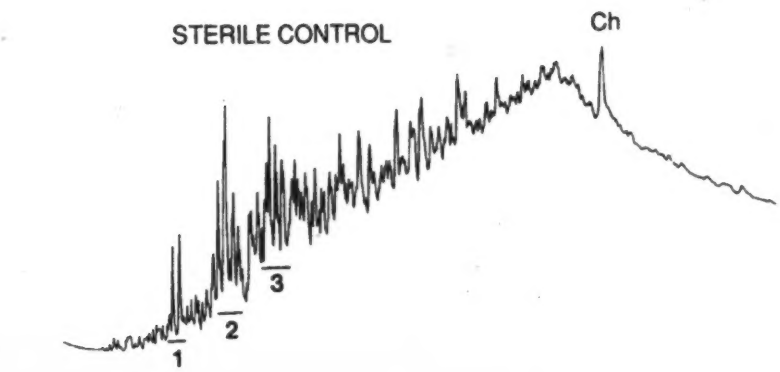
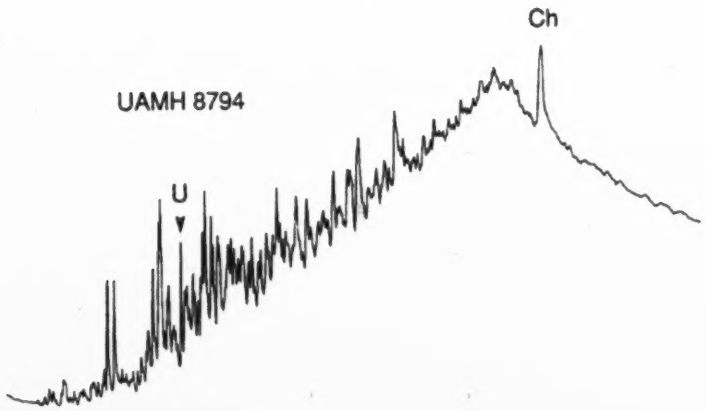

STERILE CONTROL
n-C16
n-C24
S
Pr
Ph
A
UAMH 8792
Pr
Ph
n-C24
S
B
UAMH 8793
Pr
Ph
S
C

Figure 3.7. Gas chromatographic profiles of the aromatic fraction of Prudhoe Bay Crude oil following a 21-day incubation with strains of *Pseudallescheria boydii*. **A** - Sterile control, no hydrocarbon degradation observed and similar to profiles observed with UAMH 8598 and 8793; **B** - UAMH 8794, no hydrocarbon degradation, but the appearance of a new unidentified compound. Ch, chrysene (surrogate standard); 1, 1- and 2-methylnaphthalene; 2, C_2-naphthalenes; 3, C_3-naphthalenes (Fedorak and Westlake 1981); U, new unidentified peak.



A



B

CHAPTER 4

CONCLUSIONS

In this study, filamentous fungi were isolated from hydrocarbon-contaminated soils at five flare pit sites located in western and northern Canada. Isolates were identified and tested for their ability to degrade a representative crude oil using the analytical methods of GC and radiorespirometry. It was hypothesized that there are naturally occurring fungi in hydrocarbon-contaminated soils in the regions studied and that these organisms are capable of degrading hydrocarbons. Many of the isolated fungi observed in these soils were capable of degrading hydrocarbons, based on GC and radiorespirometric studies of their growth on crude oil.

This study was successful at isolating a large number of hydrocarbon-degrading taxa, with most of the isolates belonging to the Hyphomycetes with ascomycete affinities. Few comprehensive studies have been completed that not only isolate and identify filamentous fungi from hydrocarbon-contaminated soil, but that also analyze them for their ability to degrade hydrocarbons (e.g., Oudot et al. 1987, 1993). Part of the reason may be that many of the researchers appear to have a strong microbiological and/or biochemical background to complete analytical studies of hydrocarbon degradation, but lack the mycological skills required to identify fungi.

Many of the isolates in this study belonged to the genera *Penicillium* and *Aspergillus,* which produce large numbers of small conidia. This is characteristic of many common soil fungi, and the common occurrence of these fungi may be related more to the isolation methods and media used in this study than to the isolates' relative

importance in their environment. However, *Penicillium* and *Aspergillus* species are frequent isolates of hydrocarbon-contaminated soils (Davies and Westlake 1979; Kobayashi and Rittmann 1982; Launen et al 1995; Oudot et al. 1987, 1993; Nyns et al. 1968; Wunder et al. 1994) and are known to be osmo- and xerotolerant (Dix and Webster 1995). Their ability to withstand low water availability may contribute in part to their success in this type of an environment, with their common occurrence being not just a result of the isolation methods used.

Although isolation media included selective agents such as benomyl and cycloheximide, few other species aside from hyphomycetes or ascomycetes were isolated. Considering the number of zygomycetes that have been reported in literature to degrade hydrocarbons (Chapter 1, Table 1.1), a greater number of species was expected to have been isolated. Also, no basidiomycetes were isolated or at least identified. Because many of the isolates in this study are relatively fast colonizers, the presence of basidiomycete isolates may have been masked or their development inhibited. Using a medium selective for white-rot basidiomycetes may have been useful to distinguish among them (i.e., medium containing benomyl, lignin and guaiacol; Thorn et al. 1996). There are likely many other fungi present in soil that contribute significantly to the remediation process of hydrocarbon-contaminated soils that may have been missed because of the isolation methods used.

Collections of a *Graphium* species were assigned to the teleomorph taxon *Pseudallescheria boydii* (Chapter 3). This is a significant discovery because until now, *Pseudallescheria boydii* has not been associated with hydrocarbon degradation. Other isolates assignable to the hyphomycete genus *Graphium* have been mentioned in a

number of hydrocarbon degradation studies (Lowery et al. 1968; Llanos and Kjoller 1976; Oudot et al. 1993). Although "*Graphium*" has other teleomorphic connections (i.e., *Ophiostoma, Petriella, Kernia,* and *Chaetosphaeria*; Seifert and Okada 1993; Carmichael et al. 1980), the isolates of these hydrocarbon degradation studies may in fact be related to *Pseudallescheria boydii*. This may be an indication that this species is much more wide-spread and prevalent in hydrocarbon-contaminated soils than previously known.

Many of the species that showed the ability to degrade hydrocarbons are related to ascomycete genera that can be placed into five families, as discussed in Chapter 2. This may indicate that the ability to degrade hydrocarbons is not random, but is a property of certain families, or at least certain genera. Nyns et al. (1968) tried to assign a taxonomic value to the ability to assimilate hydrocarbons in sorting taxa of filamentous fungi. They concluded that hydrocarbon assimilation is a strain-specific property and this ability cannot necessarily be reported as a property of any given species or genus. However, the substrate that those strains were isolated from was not mentioned. Strains may only exhibit this property if they had previous and/ or recent exposure to hydrocarbons. Results of the current study do indicate variability among isolates of the same species, with some isolates being capable of degrading hydrocarbons more quickly or to a greater extent than other isolates. Regardless, the ability to degrade hydrocarbons was exhibited to some degree in all isolates of the same species. Future studies should be completed on other strains in the same genera or families as those observed to degrade hydrocarbons to determine if the ability to degrade hydrocarbons is a property of these genera or families.

Many of the fungi isolated were capable of degrading hydrocarbons, as determined by GC and radiorespirometry. However, unlike the results of Kiehlmann et

al. (1996), Launen et al. (1995), Oudot et al. (1987; 1993), Sack et al. (1997) and Wunder et al. (1994), biodegradation by filamentous fungi in this study was restricted primarily to the aliphatic fraction of crude oil. The other studies tested and determined the ability of filamentous fungi, isolated from petroleum-contaminated soils, to oxidize pure PAH compounds. The exceptions were the studies of Oudot et al. (1987, 1993) which tested the ability of filamentous fungi to degrade crude oil. Results from Oudot et al. (1987, 1993) indicated that a number of isolates were capable not only of degrading compounds in the aliphatic fraction, but also compounds in the aromatic, resin and asphaltene fractions. Because fungi have the ability to hydroxylate a wide variety of compounds, fungi may be important agents in converting compounds to polar intermediates that can be further attacked by soil bacteria (Cerniglia 1997). However, one or combination of several factors may have contributed to the failure of isolates in this experiment to degrade aromatic compounds, as discussed in Chapter 2. These factors include: that none of the isolates have the necessary enzymatic systems capable of degrading aromatic compounds; most of the isolates do not perform as well in liquid culture as on agar medium; physiological stress under certain culture conditions may inhibit production of the enzymes necessary to attack the aromatic compounds or may cause an extended physiological lag time; non-static growth conditions may inhibit uptake; extended periods of incubation without hydrocarbons as a substrate may result in loss of the ability to degrade aromatic compounds; or long incubations are required for these fungi to exhibit degradation activities on aromatic compounds. Filamentous fungi are generally slower at degrading hydrocarbons than bacteria (specifically PAHs; Cerniglia 1997).

The use of GC is a common analytical technique used to determine whether organisms are capable of degrading compounds. Although GC is a relatively effective technique, radiorespirometry was also employed to determine if the biodegradation was complete (i.e., the compounds were mineralized). Many isolates were capable of mineralizing aliphatic compounds, but were incapable of mineralizing aromatic compounds. However, methods such as HPLC (as used by Kiehlmann et al. 1996 and Launen et al. 1995) could have been used in conjunction with radiorespirometry to determine if any of the aromatic compounds were hydroxylated to *trans*-dihydrodiols, phenols or other intermediates.

Certainly, more work on filamentous fungi is required to determine their role in the natural environment with respect to petroleum pollutants. This study and others like it are starting points contributing to a foundation of knowledge about microorganisms with potential as biodegraders. Fungi are most likely to behave differently in soil and in the presence of other organisms and substrates than they would as pure cultures on a Petri plate or in a liquid culture. However, information obtained from these experiments may give some indication of the processes that fungi perform in their natural environment. Oudot et al. (1993), in addition to analyzing pure cultures, did looked at the ability of mixed cultures of fungi to degrade hydrocarbons, thus taking the results obtained from pure cultures a step closer to *in situ* relevance.

Future experiments should advance from pure research to a more applied focus, and look at the ability of fungi to degrade hydrocarbons (pure or mixed) in a more natural environment. Studies may include inoculating a pseudosoil such as glass beads or acid-washed sand with microorganisms, or even using a sterilized soil. Using a sterilized soil

may indicate whether fungi preferentially degrade hydrocarbons over other carbon substrates, whether the extent and rate of degradation is affected in this environment or whether the potential to oxidize PAHs increases with other available and more readily assimilated carbon sources. Eventually, studies need to evaluate the hydrocarbon-degrading ability of fungi in the presence of other organisms also found in the soil and whether this ability is facilitated or hindered.

At this point, it is well established that fungi contribute to the biodegradation of petroleum products, but the extent of their involvement in this process under natural conditions is still undetermined. This study has in part achieved a greater understanding of species that possess specific abilities to degrade pollutants, which is an important pursuit within the larger field of bioremediation.

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APPENDIX A

SPECIES ANNOTATIONS

Below is a list of fungal species isolated from flare pit sites in western Canada. A description of each species is provided noting colony and microscopic characteristics. Where species determination is ambiguous, the closest name is given (cf.) and features are listed to support it as well as characters that bring question to its identity. Some isolates were left as an unidentified species in the form genus they most closely resembled (e.g., *Aspergillus* sp.). The isolation number of one representative isolate is provided below the name for each species. The isolation number is as follows: first letter (S or D) refers to sprinkle (direct) or dilution isolation method; next two letters refer to site (see List of Abbreviations and Symbols); first number set refers to isolation plate (e.g., 1-4-1, soil sample one - media type - isolation plate one); and last number set with letter refers to specific isolate (e.g., 1-23C).

Aspergillus fumigatus Fres.
S-WG1-4-1
1-23C
Colonies on CYA more than 35 mm diameter after 7 days, mycelium white, velutinous, conidia dull to dark green, reverse pale; conidiophores 150 - 350 µm, stipes smooth-walled; vesicles subglobose to subclavate, 20 - 35 µm; metulae uniseriate; conidia globose to subglobose, 2.5 - 3 µm, roughened.

Aspergillus niger van Tieghem
S-NW1-7-1
5-14B
Colonies on CYA more than 25 mm diameter after 7 days, white floccose mycelium with black conidia, moderate conidiogenesis; conidiophores up to 2500 µm, stipes smooth-walled, vesicles globose, 15 - 23 µm; metulae biseriate, surrounding almost entire vesicle; conidia globose, 3 - 4 µm, roughened to spinulose, dematiaceous.

Aspergillus cf. *niveus* Blochwitz
D-KS1-7-2
5-14
Colonies on CYA less than 20 mm diameter after 7 days, predominantly white with some faint yellow areas, dense and radially sulcate, clear exudate, reverse yellow gold; conidiophores smooth-walled, predominantly 200 - 300 µm; vesicles subspherical, 10 - 15 µm; metulae predominantly biseriate but some uniseriate; conidia globose, conspicuously roughed, 2.5 - 3.5 µm. Close to the species except isolates have very roughened to spinulose conidia in contrast to the smooth conidia in the description of Klich and Pitt (1988).

Aspergillus cf. *niveus* Blochwitz
D-KS2-1-2
5-14
Colonies on CYA more than 40 mm diameter after 10 days, mycelium white, floccose, radially sulcate, sparse to light conidiogenesis (more abundant at margins), white, production of yellow exudate, reverse pale yellow to tan; conidiophores, 100 - 300 µm, stipes smooth-walled; vesicles 15 - 25 µm, globose to pyriform; metulae biseriate; conidia globose, finely roughened, 3.5 - 4.5 µm. Isolate similar to other *A. niveus* isolates but mycelium almost always completely white with few yellow sectors. Also, vesicles and conidia slightly larger and reverse remaining pale to tan. Metulae almost surround entire vesicle, indicating similarity to *A. candidus*.

Aspergillus cf. *tamarii* Kita
S-KS1-6-1
5-14B
Colonies on CYA more than 50 mm diameter after 10 days, conidia olive brown, mycelium deep velutinous, radially sulcate, reverse greyish yellow; conidiophores generally more than 1 mm, stipes smooth-walled, vesicles globose (50 - 60 µm); metulae uni- and biseriate; covering almost entire surface of vesicle; conidia globose, thick, rough-walled, 3 -5 µm, dematiaceous. The isolate is very similar to *A. tamarii* in having thick, rough-walled conidia and deep brown colonies, but the vesicle diameter is larger than the 20 - 45 µm reported by Klich and Pitt (1988).

Aspergillus sp. 1
S-KS1-6-1
5-14C
Colony on CYA more than 35 mm diameter after 7 days, velutinous to floccose with raised centre, mycelium white with cream to yellow-green conidia, conidiogenesis is light to moderate; conidiophores 200 - 500 µm, stipes smooth, pigmented and thick-walled terminating in spherical to slightly elongate vesicles (14 - 16 µm); metulae biseriate, 4 - 5 µm; phialides 7 - 8 µm; conidia globose, smooth to finely roughened, 2.5 - 3 µm.

Aspergillus sp. 2
D-WG1-4-1
1-23G
Colonies on CYA more than 30 mm diameter after 7 days, mycelium white, velutinous, radially sulcate, conidiogenesis moderate, conidia pale brown, reverse pale to tan; conidiophores 100 - 250 µm, smooth-walled; vesicles subglobose to clavate, small, some 12 - 14 µm though generally < 10 µm; metulae uniseriate; phialides 5 - 6 µm; conidia globose to subglobose, smooth-walled to finely roughened, 2.5 - 3 µm. Distinguishing characteristic of this isolate is the extremely small vesicles which resemble those of *Penicillium* subgenus *Aspergilloides*. Some larger vesicles indicate this is an *Aspergillus* species.

Chaetomium globosum Kunze ex Steud.
D-DV2-1-1
8-28C
Colonies on MEA with little to no aerial growth, black perithecia readily produced and forming a dense layer; ascomata globose to subglobose, black, 150 - 300 µm; lateral and terminal pigmented hairs, terminal hairs longer, wavy, roughened; spores lemon-shaped, 9 - 11 µm x 7- 8 µm.

Cladosporium cladosporoides (Fres.) de Vries
D-KS2-1-2
5-14
Colonies on MEA slow growing, less than 2 cm diameter after 7 days, velutinous, olivaceous-green to olivaceous-brown, reverse olivaceous to black; conidiophores generally 25 - 50 µm, branching; conidia in chains, mostly lemon-shaped to ellipsoidal, some elongate, 5 - 7 (-10) µm x 3 - 5µm, mostly smooth-walled to finely roughened.

Fusarium avenaceum (Fr.) Sacc.
S-WG1-2-1
1-23D
Growth on OAT 75 - 80 mm diameter after 10 days; mycelium white and yellow-pink, floccose, reverse yellow brown and pink. Growth on PDA, 60 - 65 mm diameter, mycelium white to light pink, very floccose, reverse pinkish with dark centre. Growth on MEA, 80 - 85 mm diameter, yeast-like. Macroconidia allantoid (slender), curved, 3 - 5 septate, 30 - 50 µm in length, microconidia present, 1 septate, 12.5 - 20 µm. Sporodochia present with similar macroconidia, 40 - 55 µm.

Fusarium sp.
D-BL1-3-2
1-31D
Mycelium white, lannose to mostly floccose on OAT, reverse pale to colourless. No aerial mycelium on MEA, yeast-like with yellow-brown colour, similar reverse. Conidia 1 - 3 µm septate, slightly curved, 12.5 - 22.5 µm x 3 - 4.5 µm, apical cell curved. No microconidia. Sclerotial bodies abundant.

Fusarium subglutinans (Wollenw. & Reinking) Nelson, Toussoun & Marasas
D-DV1-2-1
8-28D
Colonies on OAT 70 - 80 mm diameter after 10 days, aerial mycelium floccose, white to pink, mostly submerged with a yeast-like appearance on agar; growth on PDA 55 - 60 mm, some pink, floccose mycelium, mostly yeast-like, brown; Mono- and polyphialides; macroconidia from aerial mycelium 1 - 3 septate, from sporodochia 3 - 5 septate, 18 - 30 (-50) µm, sickle-shaped to straight, microconidia abundant, 1 - 3 septate, reniform to oval, produced in false heads.

Geomyces pannorus (Link) Hughes
S-DV1-6-1
8-22E
Colonies on MEA white, velutinous to powdery, dense in centre, reddish-brown pigment diffusing into medium, reverse light with darkening (red-brown) center; Conidia predominantly terminal, some intercalary, hyaline, slightly echinulate, obovoid to pyriform, 4 -5 µm x 2.5 - 3 µm.

Gliocladium cf. *catenulatum* Gilm. & Abbott
S-BL1-1-1
1-31A
Colonies on MEA more than 30 mm in diameter after 10 days, mycelium white, granular to velutinous, yellow pigment diffusing into medium, reverse pale to yellow; primary verticillate conidiophores and secondary penicillate conidiophores, up to 150 µm; conidia variable in shape from broad ellipsoidal to short reniform to slightly elongate, 3 - 7 µm x 2.5 - 4 µm. Similar to *G. roseum*, but has light to intense yellow colonies whereas *G. roseum* has pink or salmon. The colour of conidia is hyaline to white from verticillate and penicillate conidiophores and not light to olive green as in the species description for *G. catenulatum*. The isolates seem to fit between the two species

Neosartorya fischeri (Wehmer) Malloch & Cain
D-NW1-4-2
5-14D
Colonies white to cream, lannose to floccose with abundant ascomata on CYA, relatively fast growing reaching 5 cm diameter in 10 days. Little mycelium on MEA but ascomata abundant. Very little conidiogenesis on MEA or CYA. Ascomata cleistothecial, white to cream, 200 - 400 µm. Ascospores broadly ellipsoidal (5 - 6 µm x 3.5 - 4.5 µm), with distinct equatorial crests that are slightly uneven, valves are spiny indicating *N. fischeri* variation *spinosa*. Conidiophores with uniseriate metulae and subglobose conidia (2 -2.5 µm), but not slightly roughened as indicated by Klich and Pitt (1988).

Oidiodendron griseum Robak.
S-BL2-2-2
1-31A
Colonies on PDA 10 mm diameter after in 10 - 14 days, grey to dark olivaceous, dark diffusing pigment sometimes present, reverse dark; conidiophores strongly dematiaceous, 40 - 150 µm, smooth-walled; arthroconidia mostly subglobose to short cylindrical, light dematiaceous, generally 2 - 2.5 µm x 1.5 - 2 µm. Some characters overlap with *O. tenuissimum* such as conidiophore size and diffusing pigment, but colony growth and colour consistent with *O. griseum*. DNA sequence supports designation as *O. griseum* (S. Hambleton, pers comm).

Paecilomyces variotii Bain.
D-DV1-2-2
8-28A
Colonies spreading broadly on MEA, yellow-green to olivaceous, deep velutinous with occasional tufts giving a powdery appearance, reverse pale to yellow brown; conidiophores repeatedly branched with whorls of slender phialides variable in size, some phialides solitary; chlamydospores present; conidia variable in shape, mostly ellipsoidal, 4 - 7 µm x 3 - 4 µm, some large with truncate base, in long chains, some larger conidia 12.5 µm x 5 µm.

Paecilomyces sp.
D-BL2-1-1
1-31C
Colonies on CYA more than 35 mm diameter after 14 days, mycelium white with light yellow center, floccose, radially sulcate, conidiogenesis sparse, reverse gold to orange; colonies on MEA velutinous, mycelium white with some yellow in centre, conidiogenesis heavy, conidia white, reverse pale to yellow; conidiophores 150 - 300 µm or more, multibranching, verticillate; metulae 7 - 8 µm; phialides divergent, slender; conidia smooth, narrowly ellipsoidal, 2.5 µm.

Penicillium cf. *aurantiogriseum* Dierckx
S-KS2-1-1
5-14D
Colonies on CYA less than 20 mm diameter after 7 days, mycelium inconspicuous, granular to floccose, conidiogenesis heavy, grey with turquoise tinge, reverse pale to golden brown; conidiophores predominantly terverticillate though some biverticillate, 200 - 500 µm, stipes lightly roughed (especially close to conidiogenous cells); conidia globose, smooth-walled, 3 - 4 µm, in long chains. The distinctive features of this isolate are the colonies which are predominantly grey with a slight turquoise tinge, and the long chains of conidia. It has a very strong and distinctive odor, but this character is not mentioned in the description by Ramirez (1982).

Penicillium cf. *decumbens* Thom
S-DV2-1-1
8-28A
Colonies on CYA more than 25 mm diameter after 7 days, velutinous, radially sulcate, conidiogenesis moderate to heavy, grey green, reverse olive brown; conidiophores monoverticillate (subgenus *Aspergilloides*), (10-) 20 - 60 µm, vesiculate or not; phialides 9 - 11 µm; conidia globose, smooth to finely roughened, 2.5 - 3 µm. Similar in colony colour to *P. decumbens* and in the short conidiophores bearing finely roughened conidia. Conidia of this isolate are distinctly globose (some subglobose) rather than ellipsoidal or pyriform as described by Pitt (1985) and Domsch et al. (1980).

Penicillium cf. *glabrum* (Wehmer) Westling
= *P. frequentans* Westling
D-DV2-2-2
8-28C
Colonies on CYA more than 30 mm diameter after 7 days, velutinous to low floccose, mycelium white to inconspicuous, conidiogenesis moderate to heavy, conidia dark grey-green, reverse pale; conidiophores predominantly monoverticillate (subgenus *Aspergilloides*) with some biverticillate, 37 - 125 µm, stipes smooth to roughened; conidia globose to subglobose, smooth, 2.5 - 3 µm. Isolates similar to *P. spinulosum* in both colony appearance and microscopic appearance but do not have spiny conidia as described by Pitt (1985). Most stipes less than 125 µm and some biverticillate.

Penicillium cf. *isariiforme* Stolk & Meyer
D-DV1-3-1
8-28B
Colony more than 40 mm diameter on CYA after 14 days, mycelium white turning yellow, reverse bright yellow, lannose to floccose, conidia white to cream, moderate on margins, mature colony forming coremia with feathery appearance; conidiophores mostly asymmetric, biverticillate, 120 - 320 µm, sometimes rami present on terminal verticils, side branches often short and monoverticillate; phialides mostly 10 - 12 µm, generally less than five per metula; conidia ellipsoidal, pointed, 2 - 3 µm. The most distinctive characteristic of this isolate is the formation of coremia that are *Isaria*-like and form conspicuous concentric patterns. Ramirez (1982) describes conidia becoming green with age, but they remain white in these isolates. However, these isolates do possess the distinctive white tips on the coremia as described by Ramirez (1982). Cultures somewhat similar to *Paecilomyces farinosus*.

Penicillium cf. *janthinellum* Biourge
D-BL2-1-1
1-31E1
Colonies on CYA 40 - 50 mm diameter after 7 days, radially sulcate, low floccose growth, mycelium white and buff, conidiogenesis light to moderate, conidia greyish-green, reverse pale yellow; conidiophores (100) 450 - 600 µm, smooth and slender, biverticillate (subgenus *Furcatum*) but some monoverticillate (generally very short), some intercalary metulae; conidia predominantly globose to subglobose, some ellipsoidal, 2.5 - 3 µm, smooth to finely roughened. Isolates tough to define but the presence of some monoverticillate coniodiophores indicates a similarity to *P. janthinellum* rather than *P. simplicissimum* although conidiophore length is more consistent with the latter.

Penicillium cf. *miczynaskii* Zaleski
D-BL2-1-1
1-31A
Colonies on CYA with more than 25 mm diameter after 7 days, velutinous, mycelium white to bright yellow, conidia with green, reverse pale; conidiophores biverticillate (subgenus *Furcatum*) with few monoverticillate, stipes 60 - 150 µm, smooth; conidia smooth, subglobose to ellipsoidal, 2.5 - 3.5 µm. This isolate resembles *P. citreonigrum* because of its short conidiophores, and *P. miczynaskii* usually has stipes 200 - 400 µm long but is known to have shorter stipes. Most of the other characteristics compare with *P. miczynskii*. Also, this isolate does not produce a yellow pigment in medium, which the species is commonly observed to do (Pitt 1985).

Penicillium cf. *minioluteum* Dierckx
S-KS2-6-1
5-14C
Colonies on CYA less than 30 mm diameter after 7 days, mycelium inconspicuous at bright yellow at margins, plane, velutinous, conidia dark green, conidiogenesis heavy, reverse dark red in centre and pale towards margin; conidiophores biverticillate (subgenus *Biverticillium*), generally 100 - 200 µm, smooth-walled; conidia ellipsoidal, smooth, 2.5 - 3.5 µm. Close to definition of *P. minioluteum* (Pitt 1985), however conidia are not narrowly ellipsoidal and colony growth rate is barely within limits. Some overlap with *P. variabile* with similar colony texture, but yellow mycelium very bright and distinct and dark green conidia also indicate isolate is more similar to *P. minioluteum*.

Penicillium pinophilum Hedgcock
S-KS2-7-2
5-14C
Colonies 20 - 30 mm on CYA, plane, funiculose to slightly floccose, mycelium white at margin with bright yellow in center, conidia dark green, reverse green to brown with some red; conidiophores biverticillate (subgenus *Biverticillium*), 100 - 200 µm, stipes smooth-walled; conidia subglobose to predominantly ellipsoidal, 2.5 - 4 µm, smooth-walled.

Penicillium cf. *purpurogenum* Stoll
or *P. rubrum* Stoll
S-NW1-2-1
5-14B
Colonies on CYA 20 - 24 mm diameter after 7 days, velutinous with raised center, plane to radially sulcate, yellow and red encrusted mycelium, heavy conidiogenesis, conidia green, reverse carmine red to very dark, no diffusing pigment into agar. Conidiophores biverticillate (subgenus *Biverticillium*), 150 - 200 µm, smooth walled; metulae 11 - 12 µm; phialides 11 - 12.5 µm; conidia narrowly ellipsoidal, smooth, 2.5 - 3.5 µm. According to Ramirez (1982), description would match *P. rubrum* because of smooth conidia, but Pitt (1985) has synonymized the two species. Encrusted hyphae are not always bright yellow or red in these cultures, which is generally a defining character of these cultures according to Ramirez (1982).

Penicillium spinulosum Thom
D-DV1-1-1
8-28B
Colonies on CYA more than 30 mm diameter after 7 days, velutinous, plane to radially sulcate, mycelium white, conidia dark green to olivaceous, reverse pale yellow to buff; conidiophores monoverticillate (subgenus *Aspergilloides*), vesiculate; stipes short, 25 - 85 µm, smooth-walled; phialides 9- 10 µm; conidia spinulose, globose, 3 - 3.5 µm.

Penicillium thomii Maire
D-WG1-1-1
1-23A
Colonies on CYA more than 25 mm diameter after 7 days, colonies velutinous to lightly floccose, radially sulcate; sclerotia numerous, apricot-coloured, produced after 14 days growth, often solitary but also in clusters of 2 or 3 (rarely), generally subglobose in shape but become irregularly shaped with maturity; conidiophores monoverticillate (subgenus *Aspergilloides*), 175 - 400 µm, rough-walled; conidia ellipsoidal, finely-roughened, 3 - 4 µm.

Penicillium cf. *variabile* Sopp.
S-KS2-7-1
5-14A
Colonies less than 20 mm diameter on CYA after 7 days, mycelium white occassionally with conspicuous yellow-green margin, conidiogenesis moderate to heavy, conidia green, reverse becoming dark red to brown but no diffusing pigment; conidiophores biverticillate (subgenus *Biverticillium*), mostly 100 - 150 µm, smooth-walled to very finely roughened; conidia smooth-walled, predominantly ellipsoidal, 2.5 - 3.5 µm. Isolates fit description of Pitt (1985) including conspicuous yellow mycelium at margins, except not all isolates produce yellow colour (sometimes remaining white to cream).

Penicillium cf. *waksmanii* Zaleski
D-BL2-4-2
1-31C
Colonies on CYA 25 mm diameter after 7 days, mycelium white with light to moderate grey-brown conidiogenesis, velutinous, radially sulcate, reverse pale; conidiophores biverticillate (subgenus *Divaricatum*) with occasional side-branches sometimes resembling short monoverticillate conidiophores; conidiophores generally 150 - 200 µm though may be much longer, smooth-walled; conidia globose to subglobose, smooth, 2.5 - 3.5 µm. Isolates relatively close to species description (Pitt 1985) but conidia colour is a more brown than green and conidiophores generally slightly longer.

Penicillium sp. 1
D-BL2-3-1
1-31B
Colonies on CYA 20 - 25 mm diameter after 7 days, mycelium white, velutinous to funiculose, radially sulcate, conidiogenesis moderate to heavy, conidia brown, reverse pale; conidiophores up to 1500 µm, terverticillate and biverticillate, usually two rami present, stipes smooth to finely roughened; metulae 7 - 9 µm; phialides 10 - 12 µm; conidia predominantly globose, smooth-walled, 3 - 3.5 µm.

Penicillium sp. 2
S-NW1-2-1
5-14B1
Colonies on CYA less than 20 mm diameter after 7 days, mycelium white and red, velutinous to fasiculate, radially sulcate, conidiogenesis moderate, conidia green, reverse light pink to red at margins (where conidiogenesis is heaviest); conidiophores biverticillate (subgenus *Biverticillium*), symmetrical, mostly 200 - 300 µm, smooth-walled; conidia ellipsoidal, smooth, 3.5 - 4.5 µm x 2 - 2.5 µm. Similar to *P. purpurogenum* with red encrusted hyphae and ellipsoidal, dark green conidia, but reverse is not dominated by dark red to amber colour, no diffusing pigment, and dominant mycelium colour is white along with red hyphae.

Penicillium sp. 3
D-DV2-6-2
8-28A
Colonies on CYA 40 - 45 mm diameter after 10 days, velutinous, radially sulcate, conidia dark green to olive green, yellow brown pigment diffusing into agar, reverse reddish-brown; conidiophores biverticillate (subgenus *Biverticillium*), smooth, 150 -200 µm; metulae 7.5 - 10 µm, phialides 11- 12.5 µm; conidia subglobose to ellipsoidal (2 - 2.5 µm), roughened.

Penicillium sp. 4
D-DV1-1-1
8-28G
Colonies on CYA more than 30 mm diameter after 7 days, velutinous, plane, white mycelium with conidia grey to slight grey-green; conidiophores bi- and terverticillate, smooth, 250 - 500 µm; conidia smooth, globose to subglobose, 2.5 - 3.5 µm.

Penicillium sp. 5
D-DV2-4-2
8-28D
Colonies on CYA 25 mm diameter after 7 days, mycelium white to inconspicuous, velutinous to floccose, radially sulcate, conidiogenesis moderate to heavy, conidia greyish-green, reverse pale yellow to beige; conidiophores biverticillate (subgenus *Divaricatum*) some monoverticillate, most less than 50 µm tall, but some greater than 100 µm; conidia ellipsoidal becoming globose to subglobose with maturity, 2.5 - 3.5 µm.

Penicillium sp. 6
D-DV1-1-1
8-28G
Colonies on CYA more than 35 mm diameter after 7 days, mycelium inconspicuous to white, velutinous to floccose, plane, conidiogenesis heavy, conidia grey green, reverse pale to yellow green; conidiophores 150 - 500 µm, biverticillate to terverticillate, asymmetrical, stipes smooth-walled; conidia produced in long columnar chains, globose, smooth-walled, 2.5 - 4 µm.

Penicillium sp. 7
D-DV2-6-2
8-28A
Colonies on CYA less than 20 mm diameter after 7 days, mycelium inconspicuous to white, radially sulcate, velutinous, conidiogenesis moderate to heavy, conidia dark grey green, reverse orange to red brown; conidiophores 125 - 200 µm, biverticillate (subgenus *Biverticillium*), symmetrical, stipes smooth-walled; conidia broad ellipsoidal to globose, smooth-walled, 2.5 - 3.5 µm.

Penicillium sp. 8
S-KS2-1-1
5-14E
Colonies on CYA more than 30 mm diameter after 7 days, velutinous, radially sulcate, mycelium inconspicuous to white, conidia grey to blue green, reverse pale yellow to light brown; conidiophores 100 - 250 µm, biverticillate with some terverticillate, stipes smooth-walled; phialides tightly packed, short and broad, 5 - 6 µm x 3 -4 µm; conidia globose, smooth-walled, 2.5 - 3.5 µm.

Penicillium sp. 9
S-KS2-1-1
5-14C
Colonies on CYA 30 mm diameter after 7 days, mycelium inconspicuous, radially sulcate, conidiogenesis heavy, conidia grey green, reverse pale to tan; conidiophores variable in size and alternate between biverticillate and terverticillate, smooth-walled; conidia globose, smooth-walled, 3 - 4 µm, borne in long, organized chains.

Penicillium sp. 10
S-BL2-5-2
1-31A
Colonies on CYA less than 20 mm diameter after 14 days, plane, center raised, velutinous to funiculose, mycelium white with light green center, conidiogenesis light, reverse pale with orange in centre; conidiophores short, 35 - 55 µm, biverticillate (subgenus *Furcatum*), stipes smooth-walled, metulae 7 - 9 µm, phialides 4 - 5 µm, conidia smooth-walled, subglobose to ellipsoidal, 2.5 - 3 µm.

Phialophora americana (Nannf.) Hughes
S-NW1-6-1
5-14F
Colonies slow-growing on MEA, woolly, olivaceous-grey to black, submerged dematiaceous hyphae, reverse black; conidiophores with solitary phialides or in groups of 2 - 3, dematiaceous; phialides 10 - 30 10 µm with very distinct collarette deeply constricted at base, collarette darker than base and occasional flaring margin; conidia, pigmented, 4 - 6 10 µm x 2 - 3 10 µm, mostly produced in sticky masses but also solitary.

Pseudallescheria boydii (Negroni & Fischer) McGinnis, Padhye & Ajello.
D-NW2-7-2
5-14C
Colonies on PDA more than 50 mm diameter after 14 days, mycelium lannose to slightly floccose when cleistothecia present otherwise floccose, grey brown, reverse pale; cleistothecia globose, 60 - 140 µm, black, on surface and submerged; asci subglobose, 14 - 16 µm x 10 - 14 µm; ascospores pigmented, smooth-walled, ellipsoidal, 7.5 - 8.5 µm x 4.5 - 5.5 µm. Only the *Scedosporium* anamorph was present; conidia hyaline to lightly pigmented, subglobose to pyriform to clavate with truncate base, 6 - 13 µm x 3 - 6 µm.

Rhizopus stolonifer (Ehrenb. ex Link) Lind
S-BL2-1-2
1-31I
Colonies on MEA fast-growing, extremely floccose, hyaline to white with dark sporangiophores and sporangiospores, reverse colourless. Sporangiophores pigmented, smooth-walled, 400 - 2000 µm x 10 - 20 µm, thick-walled, mostly in groups of 2 or 3; rhizoids pigmented, up to 400 µm long; stolons present, pigmented; sporangiospores with distinct striations, 6 - 10 µm x 4 - 7 µm, subglobose to ellipsoidal with irregular, angular edges; chlamydospores not present.

Ulocladium consortiale
D-BL1-3-1
1-31C
Colony dark, lannose, reverse dark; conidiophores dematiaceous (55 - 65 µm), geniculate; conidia not in chains, mostly with transverse septa but some with longitudinal septa, pale brown, subglobose (12.5 - 18 µm x 8 - 12 µm) to narrowly ellipsoidal (22.5 - 26.5 µm x 9 - 11 µm), most are smooth to slightly roughened.

APPENDIX B

SPECIES AND AUTHORITIES

Species	Authority
Acremonium butyri	(van Beyma) W. Gams
Acremonium kiliense	Grutz
Acremonium strictum	W. Gams
Aspergillus fumigatus	Fres.
Aspergillus niger	van Tieghem
Aspergillus cf. *niveus*	Blochwitz
Aspergillus cf. *tamari*	Kita
Beauveria bassiana	(Bals.) Vuill.
Exophiala jeanselmei	(Langeron) McGinnis & Padhye
Fusarium avenaceum	(Fr.) Sacc.
Fusarium subglutinans	(Wollenw. & Reinking) Nelson, Toussoun
Gliocladium cf. *catenulatum*	Gilm. & Abbott
Neosartorya fischeri	(Wehmer) Malloch & Cain
Oidiodendron griseum	Robak.
Paecilomyces marquandii	(Massee) Hughes
Paecilomyces variotii	Bain
Penicillium cf. *aurantiogriseum*	Dierckx
Penicillium cf. *decumbens*	Thom
Penicillium cf. *glabrum*	(Wehmer) Westling
Penicillium cf. *isariiforme*	Stolk & Meyer
Penicillium cf. *janthinellum*	Biourge
Penicillium cf. *miczynaskii*	Zaleski
Penicillium minioluteum	Dierckx
Penicillium pinophilum	Hedgcock
Penicillium cf. *purpurogenum*	Stoll
Penicillium spinulosum	Thom
Penicillium thomii	Maire
Penicillium. cf. *variabile*	Sopp
Penicillium cf. *waksmanii*	Zaleski
Phialophora americana	(Nannf.) Hughes
Phialophora verrucosa	Medlar
Pseudallescheria boydii	(Shear) McGinnis, Padhye & Ajello
Rhizopus stolonifer	(Ehrenb. ex Link) Lind
Sporothrix schenckii	Hektoen & Perkins